PASTEURELLA HAEMOLYTICA: A STUDY OF PATHOGENICITY AND IMMUNITY IN LABORATORY ANIMALS AND SHEEP

BY

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INTRODUCTION

The name Pasteurella haemolytica was introduced by Newsom and Cross (1932) and designates an organism associated in many parts of the world with disease in sheep and cattle. The first description of this organism was by Jones (1921) working in America. In a study of isolates from bovine pneumonia he discovered three groups of bacteria which could be distinguished by cultural, biochemical and serological methods. His Group I organisms consisted of Gram negative, short, non-motile, capsulated rods. Surface colonies on blood agar were round, flattened and translucent and reached a diameter of 3 to 5 mm. after 48 hours' incubation. They were haemolytic. The organisms were indol negative and produced relatively slight degrees of acidity but no gas after 7 days' incubation from dextrose, lactose, saccharose, maltose and mannitol. Only a slight local reaction resulted when rabbits were injected subcutaneously with doses of 0.25 ml. of 24 hour bouillon culture and 0.1 ml. given by the same route failed to kill mice. After a few generations on artificial media doses of 0.5 ml. administered intraperitoneally were also well tolerated by mice. The subcutaneous injection of infected bovine pleural exudate into a calf produced nothing more than a trivial local lesion and the intratracheal inoculation of 10 ml. of bouillon culture into another calf was without effect. Numerous subsequent reports have been given in the literature of bacteria whose descriptions, while differing slightly on occasions from that given above, appear to refer to organisms closely similar if not identical to those of Jones' Group I.

Organisms isolated from sheep pneumonia in America were described

by Spray (1923).

An account of the recovery of Jones' Group I bacteria from cases of bovine pneumonia in England was given by Tweed and Edington (1930). Some of their strains showed haemolysis on plates incorporating horse, rabbit and human blood. In addition to the characteristics recorded by Jones they listed the production of nitrite from nitrates and growth on McConkey medium. In typical pneumonic cases blood cultures were negative. Positive blood cultures were obtained however from two calves which died of a very severe, rapid infection. In addition to pneumonia the lesions noted in these calves included congestion and ulceration of the abomasum. Haemorrhages were present in connective tissue and were especially marked along the auriculo-ventricular groove of the heart. In the course of many attempts to infect rabbits by the intravenous, intraperitoneal and subcutaneous routes death was produced in one animal only and this by means of a large dose of about $10{,}000 \times 10^6$ freshly isolated bacteria. The intravenous injection of a Seitz filtrate of the lung juices of an affected bovine produced no signs of ill-health in a calf. A primary culture of the bacterium containing about $50{,}000 \times 10^6$ organisms was injected intravenously into a calf and produced death in 36 hours. A striking post-mortem feature consisted of numerous small subcutaneous haemorrhages. This experiment was repeated with similar results.

Dungal (1931) described a contagious pneumonia among housed sheep in Iceland. Death often ensued shortly after the onset of symptoms which sometimes included diarrhoea. On post-mortem the anterior lobes of the lung were most commonly affected and usually the

lesions were not extensive enough for death to have resulted from incapacitation of respiratory tissue alone. Affected lung resembled liver tissue and pleurisy was usually present. Sub-pleural and sub-epicardial haemorrhages constituted a common finding. The causal organism closely resembled the Group I organisms of Jones but it was said to be non-haemolytic and non-capsulated. It did not ferment lactose. It could be isolated from the blood of some but not all sick sheep, from nasal discharges and at post-mortem from affected lung tissue. Normal lung tissue was usually sterile but the organism could often be recovered from liver and heart blood and occasionally from the spleen, kidney and gall-bladder. Two mice died within 20 hours of the intraperitoneal injection of 0.5 ml. of an 18 hour broth culture but rabbits survived intravenous, intratracheal and sub-cutaneous injections of 1 ml. of 12 hour bouillon culture, and guinea-pigs survived 1 ml. given subcutaneously. Cats, dogs, hens and pigeons were not affected. A pony given 10 ml. of 12 hour broth culture intravenously showed symptoms of shivering and sweating but was normal after 3 hours. Dungal succeeded in reproducing the disease in three sheep which were housed and immediately given 1 ml. of 9 hour broth culture intratracheally. A living vaccine consisting of 12 hour broth culture was injected in doses of 1 ml. subcutaneously into each of 70 sheep. All became sick and many showed lameness. Recovery occurred within two weeks with the exception of two sheep which died and two which were killed when moribund. None of these four sheep showed advanced pulmonary consolidation but areas of collapse and haemorrhage were observed and the organism was recoverable from the lungs and heart blood. In one sheep multiple pea-sized

hepatised patches were present in both lungs. Both the living vaccine and a phenolised vaccine appeared to protect from challenge with the homologous strain. Following the use of killed vaccine in 50,000 sheep losses were negligible but no unvaccinated controls were included in the trial.

An outbreak of pneumonia among sheep in London was described by Leyshon (1932) who stated that the organism concerned closely resembled that described by Dungal.

Lovell and Hughes (1935) gave an account of 6 cases of pneumonia in calves from which a haemolytic coccobacillus was recovered in pure culture. In two cases the organism was cultured from blood in addition to lung tissue and in one case the liver, spleen and intestinal glands were also positive. Intraperitoneal injection of 1 ml. of a 24 hour broth culture failed to kill guinea-pigs, while 0.5 ml. by the same route caused death in mice in 24 to 48 hours and it was concluded that the virulence for laboratory animals was low.

Beveridge (1937) recorded a small outbreak of disease in lambs in Australia. The lesions described included pneumonia, gastritis and enteritis. Small haemorrhagic areas were present in liver tissue, the whole organ being studded with pin-point areas of necrosis. These areas were seen in histological section to contain large numbers of bacteria and there was no surrounding inflammatory reaction indicating rapid development of the condition. The organism was stated to be very similar to that described by Dungal. It was without lethal effect in mice in subcutaneous doses of up to 0.5 ml. of serum broth culture, while rabbits and guinea-pigs also resisted large doses.

An enzootic pneumonia of sheep in N. Wales and E. Anglia was described by Montgomerie, Bosworth and Glover (1938). Outbreaks were possibly precipitated by sudden environmental changes. The lesions were confined to the thorax except for an occasional suggestion of cloudy swelling in liver and kidneys. Acute cases showed extensive pneumonia, pleural exudate and haemorrhages on the pleura, pericardium and epicardium. An organism was recovered from the lungs only of 18 of 22 cases examined and was said to resemble on broad grounds that reported by Dungal. On the basis of cross-agglutination tests however it appeared to be distinct from organisms of Jones' Group I. By means of agglutination tests two sub-groups were differentiated. Rabbits tolerated doses of up to 2 ml. of 24 hour broth culture by intravenous, subcutaneous or intraperitoneal routes. Guinea-pigs and pigeons survived large doses given by a variety of routes while mice succumbed in 1 to 3 days to comparatively large doses (0.3 ml. to 0.5 ml. of 24 hour broth culture) given intraperitoneally but these strains soon lost their pathogenicity on subcultivation. The organism was without effect by other routes and mouse virulence was not appreciably altered by rapid serial intraperitoneal injections. Diseased tissue suspensions were inoculated subcutaneously, intravenously and intraperitoneally into rabbits, guinea-pigs, ferrets and mice without success. Rabbits and ferrets resisted similar inocula instilled intranasally under ether anaesthesia and injection into the lungs also failed to produce disease. A suggestion that the infection may have been established in mice by intranasal inoculation of diseased tissue suspension followed by passage was complicated by the

possible existence of latent virus infections and also by an intercurrent ectromelia infection. Pathogenicity tests in sheep were carried out using 24 hour broth cultures. Intravenous, subcutaneous and intratracheal doses produced no signs of ill-health. Negative results were also obtained using a combination of the intranasal, intratracheal and intrapleural routes. The intravenous injection of organisms contained in an agar embolus was without effect. The effect of administering infected lung suspensions to sheep was also investigated. Intranasal instillation did not produce disease and intratracheal inoculation resulted only in mild local pulmonary congestion. Animals became very ill when injected by combinations of the intratracheal and intravenous routes and of the intratracheal, intravenous and intraperitoneal routes. The lesions produced did not however resemble those seen in the natural disease; they were usually bacteriologically sterile and direct serial passage could not be achieved. Montgomerie et al. suggested that a virus might be involved together with the Pasteurella-like organism in the aetiology of the disease.

Rosenbusch and Merchant (1939) confirmed the low pathogenicity of Past. haemolytica for mice.

Bosworth and Lovell (1944) preferred not to use the name Pasteurella haemolytica and referred to organisms isolated from the nasal cavities of normal sheep and cattle as haemolytic coccobacilli. A considerable proportion of apparently normal sheep examined and a smaller proportion of cattle yielded the organism at various times of the year. Similar organisms were also recovered from nasal swabs of a proportion of sheep showing coughing and rhinitis

but it was considered that the significance of these isolates should be assessed with caution.

Campbell, Campbell and Dromey (1949) gave an account of an organism associated with an acute disease of recently housed sheep. In addition to pneumonia serous exudates, congestion of the alimentary tract and sub-epicardial petechiation were noted. A proportion of bacterial cells seemed to show ill-defined capsular material. Intraperitoneal pathogenicity tests using 24 hour broth cultures were carried out in laboratory animals. Doses of 0.5 ml. proved lethal to 2 mice, but doses of 1 ml. were resisted by 2 rabbits and 2 guinea-pigs.

Florent and Godbille (1950) described septicaemias in new-born calves and pneumonias in older animals due to an organism which was usually isolated in pure culture and whose characteristics were those of Past. haemolytica. Stress factors were considered to be of aetiological importance. It was found that of two serologically distinct types one was more frequently isolated than the other from bovine lungs. Certain strains of the commoner type produced fatal infections in guinea-pigs when injected in doses of 2 to 3 ml. of 24 hour broth culture. Peritoneal exudate collected from these animals killed guinea-pigs within 18 hours of injection of 0.1 ml. doses. The exudate also produced lethal infections in rabbits and mice but fowls and pigeons were not susceptible. Cultures inoculated intranasally produced rhinitis in sheep and one sheep which received a large dose intravenously died. Rhinitis and mild bronchitis occurred in cattle following administration of infected lung suspension intranasally and by mouth.

Pasteurella haemolytica was recovered in Norway from cases of an acute disease in young lambs and a more chronic disease in older animals by Woxholtt, Naerland and Hoff (1952). Environmental factors appeared to be concerned in the appearance of outbreaks. Lesions observed included various degrees of croupous pneumonia, enlargement of the spleen and miliary focal necrosis of the liver. A formalised vaccine was said to give a suggestion of immunity in sheep and mice.

Carter (1954) isolated Past. haemolytica from 15 of 26 young Canadian cattle which had shown the disease syndrome known as "shipping fever". Bronchopneumonia was the main lesion observed and infection was almost invariably confined to the thorax. The disease could not be reproduced in calves by inoculation of material from affected animals.

Carter and McSherry (1955) cultured Past. haemolytica from nasal swabs of 27 of 33 cattle with shipping fever.

A rapidly fatal disease of lambs in Scotland due to Past. haemolytica was described by Stamp, Watt and Thomlinson (1955). Outbreaks were usually associated with changes of pasture and could be checked by returning animals to the original grazing. The disease was septicaemic and cultures from heart blood, lung, liver, kidney and spleen yielded luxuriant growths of the organism. Lungs showed severe congestion, oedema and sub-pleural haemorrhages. Consolidation of apical and cardiac lobes was present only in cases which had survived for several days. Fibrinous pericardial fluid was present together with sub-epicardial and sub-endocardial ecchymoses. Whitish necrotic foci were visible on the surface and in the substance of the liver. The abomasum showed haemorrhagic inflammation or ulceration.

Histological findings included bacillary emboli in lung, liver and spleen. Pneumonic lesions where they occurred were considered secondary to bacterial thrombi in the pulmonary vessels. Considerable success was reported in reproducing the disease as it occurred in the field in 7 to 9 month old lambs. Inocula consisted of diseased tissue suspensions, surface culture suspensions of Past. haemolytica and broth cultures. Surface culture suspensions contained an average of 1500×10^6 organisms per ml. and were used in doses of 1 to 6 ml. Broth cultures containing $10^{8.5}$ organisms per ml. were injected in doses of 3 to 12 ml. Intravenous injections frequently resulted in death within 12 hours and the typical post-mortem appearances. Occasionally a severe polyarthritis ensued and in other cases the inoculation was without visible effect. A proportion of animals inoculated subcutaneously developed a cellulitis while others died of the typical disease within 12 hours. Intratracheal injection was without effect.

Hartley and Boyes (1955) described deaths in new-born lambs due to Past. haemolytica. Infection was presumed to have been via the navel.

Carter (1956a) found that 51 strains of Past. haemolytica isolated from shipping fever in Canada were serologically homogeneous by means of a haemagglutination test. Strains from America, Europe and two from cases of lamb septicaemia in Scotland were judged to be identical to the Canadian strains by haemagglutination and conventional agglutination tests.

Carter (1956b) claimed to have been successful in establishing infections in a proportion of calves by a variety of methods. The

most severe infections were in animals which appeared to have had inapparent chronic pneumonia prior to exposure.

Stevens (1957) gave statistical evidence of the importance of Past. haemolytica as a cause of sheep pneumonia in Great Britain and considered that a virus was unlikely to be involved in the aetiology of the disease. The importance of environmental factors was stressed however. The use of formolised broth culture vaccines in dealing with a number of outbreaks gave some encouragement but few of the trials included controls.

Salisbury (1957) described enzootic pneumonia of sheep in New Zealand. Bacterial cultures, diseased lung suspensions and combinations of cultures and diseased lung filtrates were tested in sheep of all ages by the intranasal, intratracheal and intrapulmonary routes. The results were invariably negative. It was suggested that the primary aetiological agent might be a virus and that failure to produce the disease by inoculating tissue suspensions was possibly the result of using material which was not fresh.

Downey (1957) also working in New Zealand described cases of enzootic pneumonia in sheep associated with Past. haemolytica infection. Fresh tissues were frozen and sent to the laboratory without delay for examination. Considering that a virus might also be involved Downey attempted to transmit such an agent to embryonating hens' eggs. Experimental inoculations of 43 sheep were carried out using a variety of routes. Two partially successful transmissions were reported using combinations of the intravenous and intratracheal routes. One of the sheep received diseased lung suspension and the other an inoculum composed of Past. haemolytica culture together with

chorio-allantoic membrane suspension from an egg presumed to be infected with virus. In his paper Downey referred to a personal communication from Hartley and Boyes to the effect that intravenous or intraperitoneal inoculations of new-born lambs with Past. haemolytica resulted in a rapidly fatal infection simulating a naturally acquired neonatal disease. Older lambs and sheep were resistant to intraperitoneal injections.

Gale and Smith (1958) failed to infect 4 calves exposed to Past. haemolytica by a variety of methods, one of which included cortisone treatment.

Biberstein, Meyer and Kennedy (1958) reported colonial variation of a strain of Past. haemolytica isolated from a lamb septicaemia. The strain was reported to be pathogenic for guinea-pigs. The variant colony was only faintly grey and its surface was finely textured. It possessed an affinity for crystal violet stain. Apparently organisms from standard and variant colonies showed no clear-cut morphological differences and were identical biochemically. Lambs were equally susceptible to inoculations of both types but mice were said to succumb more readily to injections of the standard type. It was concluded from the results of serological tests that the two types possess different amounts of a soluble surface antigen.

Hamdy and Pounden (1959) reported that a broth culture of Past. haemolytica injected into a lamb by a combination of intranasal and intratracheal routes failed to produce any effect.

In an examination of throat swabs taken from ewes and their lambs Hamdy, Pounden and Ferguson (1959) found that 25 of 41 lambs and 18

of 36 dams yielded Past. haemolytica. The organism was recovered from 9 of 51 lambs which showed pneumonic lesions at post-mortem.

Past. haemolytica septicaemia of lambs in California was reported by Biberstein and Kennedy (1959). Bacterial colonies were present in lungs, liver, spleen and adrenal cortex but there was no well-marked associated inflammatory reaction. Fatal infections of guinea-pigs were produced by intraperitoneal and intracardiac inoculations of doses varying from 20×10^6 to 500×10^6 organisms. Intraperitoneal inoculations resulted in fibrinous peritonitis and smears of exudate showed enormous numbers of distinctly encapsulated bacteria. Intranasal instillation of 1000×10^6 organisms gave negative results. Mice were approximately as susceptible as guinea-pigs to experimental infection, but young hamsters, chickens and rabbits were resistant. The naturally occurring disease was reproduced in a proportion of lambs inoculated intravenously with large doses of organisms. Polyarthritis was produced in several cases. Intranasal inoculation of lambs treated with corticosteroids was unsuccessful.

This review of the literature shows the difficulties associated with the experimental production of Past. haemolytica infections, particularly enzootic pneumonia, in sheep. These difficulties together with the high cost of the sheep as an experimental animal have severely retarded work on immunity. Indirect immunological studies using laboratory animals have not been pursued since the experience of most workers has been that Past. haemolytica is of low pathogenicity for these animals. These problems have provided the stimulus for work now to be described. The thesis consists of a

study of pathogenicity and immunity in laboratory animals and sheep and includes observations on two distinct types of Past. haemolytica which appear to be concerned with the production of different forms of disease in sheep.

PART 1

EXPERIMENTAL INFECTIONS OF PASTEURELLA HAEMOLYTICA IN MICE AND THEIR USE IN DEMONSTRATING PASSIVE IMMUNITY

In the past the apparent lack of a suitable experimental animal has severely curtailed studies of Past. haemolytica. A description is given here of two methods by means of which true infections can be established in mice, with doses considerably smaller than hitherto, characterised by marked multiplication of the organism and affording a means of demonstrating protection by immune serum. These two methods are (a) intracerebral injection, and (b) intraperitoneal injection using granular mucin as an aid to infection.

MATERIALS AND METHODS

Strains of Past. haemolytica. All strains used in the following experiments were isolated from sheep in Scotland with the exception of No. 32 which was obtained from the pneumonic lung of a lamb in S. Wales. Nos. 8 and 36 were isolated from cultures of nasal swabs taken from apparently healthy sheep (No. 36 was the predominating organism from the swab). No. 34 was from a severe case of pneumonia in an adult sheep, and Nos. 1118 and 7157H were cultured from the blood and organs of two lambs which died from a septicaemic condition. These six strains were the only ones examined in any detail, but from more empirical observations on a considerable number of other strains they appeared to be typical as regards their mouse pathogenicity. No. 7157H was the only strain used for protection experiments and it was selected at random for

this purpose. A culture made from the heart blood of an experimentally infected mouse was freeze-dried for use in the intraperitoneal protection tests. This culture was also used for the comparative titrations of living and heat killed organisms. For challenge purposes in intracerebral protection experiments No. 7157H was freeze-dried after the forty-second consecutive intracranial mouse passage.

Bacterial suspensions for inoculation. Unless stated otherwise, all inocula consisted of 18 to 20 hour growths on 5 per cent sheep blood agar, scraped off and evenly suspended in a solution of 1 per cent casein hydrolysate in distilled water, pH 7.1. These suspensions were then suitably diluted in casein hydrolysate solution and in the case of intraperitoneal experiments mixed with mucin. The temperature in all instances was maintained at 4°C until shortly before the time of injection. Wherever it was necessary to inject mice with a reasonably accurate dose of bacteria, advantage was taken of the fairly constant low death rate of Past. haemolytica in 1 per cent casein hydrolysate solution at 4°C. It was found that, in general, a loss in viability of about 25 per cent could be expected when suspensions were maintained under these conditions for 24 hours. Accordingly, a suspension could be prepared on Day 1 and a viable count made. The result of the count would be available on Day 2 (the day of inoculation) and the suspension could then be diluted to contain the required number of living bacteria per millilitre, allowing for a 25 per cent loss in viability. The results of a further viable count on this adjusted suspension performed just before inoculation would then be available on Day 3 as

a retrospective check. In practice, this method was quite satisfactory and was used in all the following experiments with the exception of the comparative titrations of living and heat-killed suspensions and of the intracerebral virulence titrations. In these experiments the organisms were harvested from blood agar and inoculated on the same day.

Viable counts. These were performed by the method described in Appendix II. Five replicate counts were carried out in each case and the average taken as the true count.

Sera. Hyperimmune serum against strain No. 7157H was prepared in two rabbits, each of which received a course of intravenous injections of formalin-killed bacteria, followed by increasing doses of living organisms. A proportion of these injections consisted of organisms which had been passaged intracerebrally a number of times in mice. The maximum immunising dose used was 0.45 ml. of living 24 hour broth culture. Agglutinin titres were followed in these rabbits using a stock formalinized suspension of strain No. 7157H, diluted to Brown's tube 3.5 opacity before use. Agglutination tests were incubated at 55°C for 2 hours and all protection experiments were performed after the two sera had reached their maximum titre, which in both cases was 1/1024. Normal rabbit sera, checked for absence of agglutinins, were used for control mice. All sera were sterilized by filtration and were used two days after collection from the rabbits. None was inactivated by heat. Serum dilutions were made up in normal saline and were injected intraperitoneally, in a dose volume of 0.5 ml., 5 to 6 hours

before challenge.

Mice. Young Swiss white mice from the Moredun Institute closed colony were used. Those for intracerebral inoculation weighed from 15 to 20 gm., while those used in all other experiments were somewhat larger, ranging from 17 to 22 gm. The sexes were evenly distributed.

Intracerebral inoculations. Injections of organisms suspended in casein hydrolysate solution were made under ether anaesthesia. Doses of 0.05 ml. were delivered from a 0.25 ml. syringe with a 26-gauge needle.

Intraperitoneal inoculations incorporating mucin. Granular mucin, type 1701-W*, was made up at the rate of 5 gm. of mucin per 100 ml. of distilled water, and was prepared, autoclaved and neutralized according to the manufacturer's instructions. This mucin suspension was used in the proportion of 4 parts of mucin to 1 part of bacterial suspension. The dose volume per mouse of this inoculum was standardized at 0.5 ml. Provided that organisms were omitted, such an inoculum was shown to be well tolerated by mice. Dilution series of organisms for intraperitoneal inoculations were never prepared by using mucin suspension as the diluent for fear of errors, due to mixing in a rather viscous fluid, being transmitted down the series. Instead, dilution series were prepared in casein hydrolysate solution, a measured volume from each dilution tube being transferred to 4 times this volume of mucin suspension and mixed very thoroughly with a pipette. Where parallel control mice were to be

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* Wilson Laboratories, Chicago, U.S.A.

inoculated with organisms only and not mucin, as in the experiments designed to demonstrate the capacity of mucin for reducing the resistance of mice to Past. haemolytica, a measured volume from each dilution tube was transferred into 4 times this volume of casein hydrolysate solution. The dose volume of such control inocula was again 0.5 ml.

RESULTS

(a) Virulence and toxicity of intravenous and intraperitoneal inocula

Preliminary observations on mice confirmed the conclusions of earlier workers as to the low virulence of Past. haemolytica when administered by conventional methods. Lethal infections in mice could be produced only by exceedingly large doses of bacteria and it seemed probable that deaths in such cases might well be due in part to the toxicity of these large inocula.

To investigate this point an experiment was performed in which a heavy suspension of Past. haemolytica containing 33.8×10^9 viable organisms per millilitre and having the opacity of Brown's Tube 100 was titrated in 5-fold dilutions both in the living state and after sterilisation by heat at $56^{\circ}C$ for 30 minutes. Comparable inocula were administered by the intravenous and intraperitoneal routes. The dose volume per mouse was 0.5 ml. The results are recorded in Table 1. Even the heaviest inocula were well tolerated initially and 10 minutes after the completion of inoculations all mice were fairly bright. Within 2 hours however 4 out of 6 mice which received the largest dose of living organisms intravenously were dead. The majority of deaths occurred within 24 hours. The experiment

TABLE 1

COMPARATIVE INTRAVENOUS AND INTRAPERITONEAL TITRATIONS OF LIVING AND HEAT KILLED PAST. HAEMOLYTICA (STRAIN 7157H) IN MICE

Dilution of inoculum	Deaths in groups of 6 mice treated as shown			
	Inoculated intravenously		Inoculated intraperitoneally	
	Viable inoculum	Heat killed inoculum	Viable inoculum	Heat killed inoculum
1/1	6	6	6	6
1/5	5	0	6	0
1/25	0	0	5	0
1/125	0	0	0	0

Undiluted inocula were equivalent in opacity to Brown's Tube 100 and unless sterilised by heat contained 16.9×10^9 viable organisms.

Dose volume per mouse = 0.5 ml.

was discontinued on the sixth day after inoculation at which time all survivors appeared healthy. The results recorded in the Table show that the lethal dose of living bacteria comprised a low multiple of the lethal dose of heat killed bacteria and this was particularly so where inoculations were made intravenously. It is also apparent that living organisms produced deaths more readily when injected intraperitoneally than intravenously.

(b) Intracerebral inoculation

All strains of Past. haemolytica tested have proved capable of producing true infections of mice by the intracerebral route. Experiments have shown that mice destined to die almost always do so within 10 days, although very occasionally deaths occur up to the 14th day. The symptoms are typical of a cerebral infection and at death the organisms are usually confined to the brain and meninges, although occasionally a loopful of heart blood will give rise to one or two colonies when cultured on blood agar. Smears from brain tissue generally reveal very large numbers of bacteria on microscopic examination. Pleomorphism is usually either absent or slight. Counts of viable organisms in brain tissue suspensions, prepared by disintegrating whole weighed brains in measured volumes of 1 per cent casein hydrolysate solution in Griffith tubes, reveal that multiplication occurs to a high degree. For example, the brain of one mouse which died on the 6th day after receiving an estimated dose of 230 organisms contained 12.2×10^6 viable bacteria at death. The brain of another mouse contained 2000×10^6 viable organisms at death which occurred within 24 hours of the injection of 2.75×10^6 organisms.

Table 2 shows the results of virulence titrations of five different

TABLE 2

INTRACEREBRAL VIRULENCE TITRATIONS OF 5 STRAINS OF PAST. HAEMOLYTICA IN MICE

Strain No.	Dose of viable organisms	Showing the days after inoculation on which each individual mouse died												Total deaths
8	10×10^6	1	1	1	1	2	3							6/6
	10×10^4	2	3	4	4	4	4	5	5	6	7	S	S	10/12
	10×10^2	2	3	S	S	S	S	S	S	S	S	S	S	2/12
	10	2	2	S	S	S	S	S	S	S	S	S	S	2/12
	0	S	S	S	S	S	S							0/6
32	46×10^6	1	1	1	1	2	4							6/6
	46×10^4	3	4	5	5	5	6	7	S	S	S	S	S	7/12
	46×10^2	1	3	3	5	5	S	S	S	S	S	S	S	5/12
	46	4	4	4	4	X	S	S	S	S	S	S	S	4/11
	0	S	S	S	S	S	S							0/6
34	17×10^6	1	1	1	2	2	2							6/6
	17×10^4	1	1	2	4	4	4	5	5	6	6	S	S	10/12
	17×10^2	2	3	3	3	4	S	S	S	S	S	S	S	5/12
	17	6	S	S	S	S	S	S	S	S	S	S	S	1/12
	0	S	S	S	S	S	S							0/6
36	83×10^6	1	2	2	2	5	6							6/6
	83×10^4	1	2	4	4	6	6	7	S	S	S	S	S	7/12
	83×10^2	1	1	1	2	5	5	6	S	S	S	S	S	7/12
	83	1	2	S	S	S	S	S	S	S	S	S	S	2/12
	0	S	S	S	S	S	S							0/6
7157H (Passaged)	57×10^6	1	1	1	1	1	2							6/6
	57×10^4	1	2	3	3	3	4	5	6	6	S	S	S	9/12
	57×10^2	1	1	3	3	3	3	3	4	4	S	S	S	9/12
	57	3	4	4	5	7	S	S	S	S	S	S	S	5/12
	0	S	S	S	S	S	S							0/6

S = Mouse survived.

X = Mouse died but brain sterile.

strains. With the exception of No. 7157H which was tested immediately after the 34th consecutive intracerebral mouse passage, these strains had been maintained in artificial culture for varying periods in the laboratory. It will be seen that the susceptibility of mice varies considerably, some dying after a dose of less than 100 viable organisms, others resisting a dose 10,000 times as great. With the numbers of mice used even 100-fold dose differences frequently resulted in little or no change in mortality. The LD_{50} would in general appear to be in the low thousands.

(c) Modified method of producing cerebral infections. This modification is similar to that used by Sawyer and Lloyd (1931) in their work on yellow fever.

The present investigations have shown that a sub-lethal intravenous dose of Past. haemolytica administered to a mouse can be converted into a lethal dose by subsequent brain trauma. Injury to the brain can be effected by a number of treatments, one of which consists of the intracerebral injection of 0.03 ml. of 2 per cent starch-saline solution. Such a treatment is, in itself, well tolerated by mice. Mice die within 10 days showing symptoms of a cerebral infection and at post-mortem smears of brain tissue show very large numbers of bacteria. Frequently culture indicates that organisms are confined to the brain, but in other cases considerably smaller numbers may be cultured from heart blood and spleen also.

It appears to be preferable to use a strain which has been passaged intracerebrally a number of times in mice for this method of infection. Using such a strain in a suitable dose it is possible to obtain a mortality of 100 per cent as compared with no deaths in

control mice receiving identical intravenous doses but whose brains are left intact. It is essential that the time interval between injection of organisms and brain injury should not be too long and this is illustrated by the experiment recorded in Table 3. Mice were inoculated intravenously with broth culture and sterile starch-saline solution was injected intracerebrally at various time intervals afterwards. No deaths occurred if the starch injection was delayed for as long as 2 hours, by which time presumably many bacteria had been removed from the circulation.

(d) Intraperitoneal inoculation using granular mucin

Mucin preparations have been used in the past for reducing the resistance of experimental animals to a number of micro-organisms whose virulence is such that inordinately large numbers of bacteria are necessary to produce lethal effects. Much of this work has been reviewed by Olitzki (1948).

The present study has shown that the numbers of Past. haemolytica required to produce fatal infections in mice by intraperitoneal injection are markedly reduced if mucin is incorporated in the inoculum. A rapidly fatal septicaemia results, the great majority of deaths occurring within 48 hours of inoculation. Smears of peritoneal fluid and heart blood of dead mice generally show very large numbers of bacteria per field on microscopic examination. Usually the organisms are quite regular in shape and size, but smears from a particular mouse occasionally show very marked pleomorphism, which may take the form of grossly thickened or elongated bacteria or globular forms similar to those of H. influenzae described by Pittman (1931).

TABLE 3

DEATHS IN MICE RECEIVING INTRAVENOUS DOSES OF PAST. HAEMOLYTICA FOLLOWED BY INTRACEREBRAL INJECTION OF STARCH SOLUTION AFTER VARIOUS INTERVALS

Dose of 6 hour broth culture intravenously	0.03 ml. of 2% starch solution intracerebrally	Interval between intravenous and intracerebral injections	Deaths in groups of 6 mice
0.4 ml.	+	<5 mins.	6
0.4 ml.	+	30 mins.	3
0.4 ml.	+	60 mins.	2
0.4 ml.	+	120 mins.	0
0.4 ml.	-	-	0

Strain of Past. haemolytica used was No. 7157H after 31 intracerebral mouse passages.

TABLE 4

INTRAPERITONEAL TITRATIONS
5 strains of Past. haemolytica in mice,
with and without the use of mucin

Strain No.	Mucin	Deaths in groups of 4 mice using dilutions of organisms shown below								
		Maximum dose	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
32	-	1	0	1	1	N	N	N	N	N
	+	N	4	4	2	1	1	0	0	0
34	-	1	0	0	0	N	N	N	N	N
	+	N	4	3	2	3	1	0	0	0
36	-	2	2	1	0	N	N	N	N	N
	+	N	4	3	4	4	3	2	0	0
1118	-	2	1	0	0	N	N	N	N	N
	+	N	4	2	3	2	1	1	1	0
7157H	-	4	1	0	0	N	N	N	N	N
	+	N	4	2	2	0	0	0	0	0

N = Not done.
Viable counts showed maximum doses to contain the following numbers of organisms

Strain	32	1030×10^6
"	34	1125×10^6
"	36	1090×10^6
"	1118	1030×10^6
"	7157H	1025×10^6

Table 4 shows the results of titrations of 5 strains over a wide range of decimal dilutions, with and without the use of mucin. Cultures were made from each dead mouse to confirm that it had died from a pure infection of Past. haemolytica. The largest inoculum of each strain was made to contain approximately 1000 x 10^6 viable organisms in order that rough comparisons between strains might be possible. It will be seen that mucin lowered the resistance of mice to all strains tested, but to a greater degree for some than others. The very large dose necessary to produce deaths in mice not receiving mucin is also demonstrated.

That multiplication of the organism occurs when it is injected intraperitoneally together with mucin was conclusively demonstrated by the rapid serial passage of strain No. 7157H direct from mouse peritoneum to peritoneum. Peritoneal washings in casein hydrolysate solution were collected from dead mice, and a small portion of each washing was injected intraperitoneally together with mucin into each of two mice, one of which received a rather larger inoculum than the other. In this way it was always possible in practice to post-mortem at least one animal shortly after death, thus avoiding contamination. Inocula were regularly checked for purity by aerobic and anaerobic culture. Twenty-four consecutive mouse passages were obtained without difficulty. After these 24 passages it was no longer necessary to incorporate mucin in the inoculum and a further 24 passages were achieved by injecting small portions of peritoneal washings alone, after which the experiment was discontinued. It appeared that for this adaptation to the mouse peritoneum the use of mucin in the early passages was essential. Three subsequent

attempts to so adapt the original strain 7157H without the use of mucin failed. The initial inocula were massive doses of broth culture and large amounts of each peritoneal washing were used as inocula. However, the best that was achieved in this way was 5 consecutive passages before the organism died out.

(e) Intracerebral protection tests

In the first of these experiments groups of mice received various dilutions of hyperimmune rabbit serum intraperitoneally. A further group received normal saline intraperitoneally in comparable volumes. Each mouse was subsequently challenged with Past. haemolytica injected intracerebrally. Deaths were recorded in each 24 hour period, and the results are shown in Table 5. It is obvious from the trivial differences between the final number of deaths in each group that the effect of immune antibody on actual survival of mice in this experiment was slight if present at all. However, considering survivals on the second and third days it would again seem that 0.5 ml. of undiluted rabbit serum has lowered the resistance of mice to the infection and this is shown in the experiment as a more rapid death rate in these mice than in those receiving 1/5 serum. In the same way it will be seen that 1/5 hyperimmune rabbit serum tends to prolong survival time as compared with saline, but this protective effect is rapidly diluted to extinction.

Figure 1 illustrates the results of a protection test in which the effect of a constant dose of serum on graded challenge doses was studied. One hundred and twenty-six mice were inoculated intraperitoneally with 0.5 ml. of 1/5 hyperimmune rabbit serum, this dose

TABLE 5

SURVIVAL TIMES IN MICE RECEIVING DILUTIONS OF HYPERIMMUNE RABBIT SERUM PRIOR TO INTRACEREBRAL CHALLENGE WITH PAST. HAEMOLYTICA

		Showing the day after challenge on which each individual mouse died																		Final deaths in groups of 18 mice
Dilution of immune serum	1/1	1	2	2	2	2	2	2	2	2	3	3	3	4	4	4	7	S	S	16
	1/5	1	1	2	3	3	3	4	4	4	4	4	4	10	S	S	S	S	S	13
	1/10	1	1	2	2	2	3	3	3	3	3	3	4	4	4	4	S	S	S	15
	1/20	1	1	1	1	2	2	2	2	3	3	3	3	4	4	5	S	S	S	15
Normal saline		1	1	1	1	2	2	2	2	2	3	3	3	3	3	4	4	S	S	16

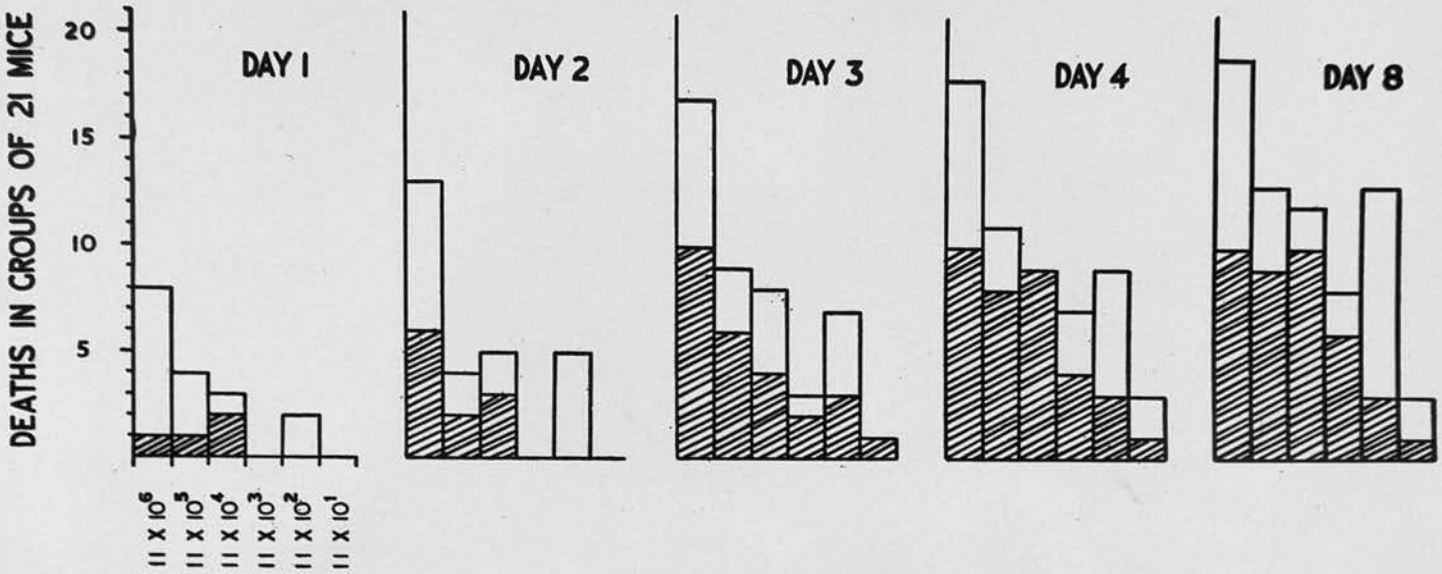
Dose volume of serum dilutions and saline = 0.5 ml. intraperitoneally.

Strain of Past. haemolytica used was No. 7157H after 42 intracerebral mouse passages.

Challenge dose per mouse = 0.85×10^6 viable organisms.

S = Mouse survived.

Fig. 1



Effect of graded intracerebral doses of *Past. haemolytica* on mice pretreated with hyperimmune and normal rabbit serum.

Dose of appropriate serum per mouse = 0·5 ml. of 1/5 dilution intraperitoneally.

Strain of *Past. haemolytica* used was No. 7157H after 12 intracerebral mouse passages.

Shaded areas represent total deaths to date in mice protected with immune serum. These are superimposed on clear areas which represent total deaths in control mice inoculated with normal serum.

Deaths in protected mice did not at any time or any challenge dose exceed deaths in control mice.

No deaths occurred after the 8th day.

being chosen from a consideration of the previous experiment. These mice were then divided into 6 equal groups which were subsequently challenged intracranially with 10-fold dilutions of organisms. One hundred and twenty-six control mice were treated in an exactly similar manner except that normal rabbit serum was substituted for hyperimmune. Deaths were recorded on each day after challenge. No deaths occurred after the 8th day. Although the protection afforded by immune serum against this method of challenge was not dramatic, a comparison of survivors among protected and control mice on each day did in fact demonstrate protection. The most striking comparison was obtained at the end of the first day.

(f) Intraperitoneal protection tests using mucin

The objects of this part of the work were to show (1) that a hyperimmune rabbit serum could be prepared which would protect mice against intraperitoneal challenge with Past. haemolytica in mucin; (2) that such a serum could be titrated in mice against a constant challenge dose, and (3) the effect of using graded challenge doses against a single dose of serum.

Preliminary experiments indicated the approximate range of challenge dose of strain No. 7157H over which immune antibody effects might be demonstrated. This work was repeated subsequently by a somewhat modified experiment, the results of which are recorded later. Initially, however, it was desirable to gain at least a rough knowledge of that dose of immune serum which would be large enough to produce a high level of immune antibody in mice consistent with the elimination of any undesirable attributes of

rabbit serum which might become manifest if too large a dose were used.

Information on these points was supplied by an experiment the results of which are shown in Table 6. Groups of mice received varying doses of hyperimmune and normal rabbit serum prior to challenge with organisms in mucin. The experiment was duplicated using a second challenge dose half the size of the first. This reduced the risk of supplying an unsuitable dose of organisms due to technical errors, a risk which might be very real if only a single dose level were used. Thus one could choose to interpret the test either on the basis of deaths in mice at single challenge levels or on the basis of deaths at both levels combined, whichever might be more advantageous. The practical benefits of this procedure are, in fact, better demonstrated in the next test to be described. Deaths were recorded 24 hours after challenge and again at 72 hours, after which time no further deaths occurred. The results show that 0.5 ml. of undiluted normal rabbit serum is capable of providing protection to mice as compared with 0.5 ml. of 1/100 immune rabbit serum, but under the conditions of the experiment this effect is manifested as a prolongation of survival time and tends to be obliterated as the test progresses towards completion. At the same time it is apparent from a comparison of final deaths of mice receiving 1/1 and 1/4 immune serum that 0.5 ml. of undiluted rabbit serum also exerts an eventual lethal effect. This effect is probably better thought of as decreasing the resistance of mice to the experimental infection since 10 mice which were subsequently given a similar intraperitoneal dose of rabbit serum alone merely showed a

slight loss in weight from which they rapidly recovered. It would seem from these results that in choosing a single dose of hyperimmune rabbit serum for the purpose of demonstrating protection in mice under optimal conditions it would be unwise to exceed 0.5 ml. of a 1/4 dilution, and, indeed, quite unnecessary since a 1/14 dilution is also adequate.

Table 7 shows the results of titration of hyperimmune rabbit serum in mice. The method used was similar to that of the previous experiment except that the groups of mice were smaller. A control titration of normal rabbit serum was performed in parallel and under identical conditions. Mice receiving 0.5 ml. of saline instead of serum prior to challenge were also included. The upper challenge level of 90×10^6 viable organisms in mucin was capable of demonstrating protection by immune serum only as far as the 1/14 dilution. The smaller challenge dose however was more successful, and combining the deaths obtained using it against both the 1/100 and 1/200 dilutions of sera it is clear that protection was still present at the 1/100 dilution of immune serum.

The results of the final experiment in this section are recorded in Table 8. The purpose in this case was to study the effect of using graded challenge doses of organisms in mucin against a constant dose of serum. Each challenge level was tested against normal and hyperimmune rabbit serum. Serum was injected intraperitoneally in volumes of 0.5 ml. of a 1/10 dilution, this dose being chosen on the basis of information provided by experiments already described. Organisms were injected in 2-fold dilutions since effective challenge doses were known to be high. The immune serum gave a very high

TABLE 7

COMPARATIVE TITRATIONS OF HYPERIMMUNE AND NORMAL RABBIT SERA IN MICE

Intraperitoneal challenge with Past. haemolytica (Strain 7157H)

Serum dilutions		1/4		1/14		1/50		1/100		1/200		
Type of serum		I	N	I	N	I	N	I	N	I	N	Saline
Deaths in groups of 10 mice using 2 challenge levels	90×10^6 organisms	2	9	0	8	7	10	9	9	9	8	9
	45×10^6 organisms	0	5	1	8	1	10	5	10	5	10	10
Total deaths		2/20	14/20	1/20	16/20	8/20	20/20	14/20	19/20	14/20	18/20	19/20

I = Hyperimmune rabbit serum.

N = Normal rabbit serum.

Dose volume of serum dilutions = 0.5 ml. intraperitoneally.

Challenge inocula incorporated mucin.

TABLE 8

1/10 DILUTIONS OF HYPERIMMUNE AND NORMAL RABBIT SERA

Graded intraperitoneal challenge doses of Past. haemolytica (Strain 7157H) in mice

	Deaths in groups of 12 mice using challenge doses shown below				
	140×10^6	70×10^6	35×10^6	17.5×10^6	8.75×10^6
1/10 immune serum	4	0	1	0	0
1/10 normal serum	12	11	10	1	0

Dose volume of 1/10 serum = 0.5 ml. intraperitoneally.

Challenge inocula incorporated mucin.

degree of protection over a particular range of challenge, the order of which is indicated by the Table. It is worthy of note that this experiment was performed on the same day as was the one which is recorded in Table 5, using the same batch of hyperimmune serum.

PART 2

THE PRODUCTION IN MICE OF ACTIVE IMMUNITY AGAINST PAST. HAEMOLYTICA

The work already described was extended to include a study of the protective effects of vaccination of mice against intraperitoneal challenge with organisms suspended in mucin. The results of these investigations are now recorded.

MATERIALS AND METHODS

Details of certain technical procedures and materials used have already been given. They cover the preparation and adjustment of bacterial suspensions for inoculation, the method of performing viable counts and the method of intraperitoneal challenge using organisms and mucin.

The same strain of Past. haemolytica (No. 7157H) originally isolated in 1954 from a lamb which died of a Past. haemolytica septicaemia as described by Stamp, Watt and Thomlinson (1955), was used for all vaccination and challenge purposes in the present series of experiments. A culture made from the heart blood of an experimentally infected mouse was freeze-dried in ampoules which were stored and used as required.

Preparation of vaccines.

Living vaccine. This consisted of a 24 hour infusion broth culture injected in a dose volume of 0.2 ml. of a 1/5 broth dilution. Such a dose was known to be sub-lethal for mice although it contained 40 to 50 million viable bacteria.

Formol-saline vaccine. Seventeen-hour cultures on 5 per cent

sheep blood agar were suspended in normal saline and sterilised by washing twice and resuspending to the opacity of Brown's Tube 6 in 0.5 per cent formol-saline. Suitable tests for purity and sterility were included. A single batch of this vaccine was prepared and stored at +4°C for use in all the following experiments. The dose volume per mouse was 0.2 ml.

Adjuvant vaccine. The method of preparation consisted of a modification of the procedures used by Live (1949). A portion of the formol-saline vaccine described above was treated as follows. To each 1.0 ml. volume was added 0.027 ml. of sterile 10 per cent sodium bicarbonate solution and 0.25 ml. of sterile 4 per cent potassium aluminium sulphate solution. The mixture was allowed to stand overnight at +4°C, after which the flocculated bacteria were washed and resuspended in 0.5 per cent formol-saline. One volume of this suspension was then added to 4 volumes of sterile oil-Falba mixture which itself consisted of Bayol F* and Falba** in the proportion of 4 volumes to 1 volume. This final mixture was thoroughly emulsified. The dose per mouse was 0.05 ml. and the concentration of the vaccine was such that this volume contained the same number of dead bacteria as did a single dose of the formol-saline vaccine.

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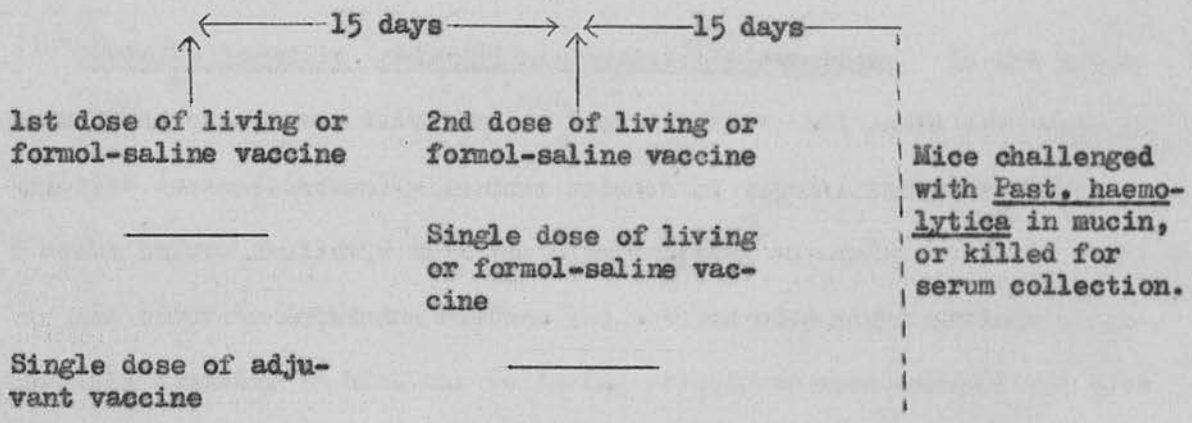
* Bayol F is a light mineral oil supplied by the Esso Petroleum Co. Ltd.

** Falba is a lanolin-like substance supplied by Pfaltz and Bauer, Inc., New York, U.S.A.

Vaccination and challenge schedules. Swiss white mice were used and at the beginning of each experiment they were 4 to 5 weeks old. The sexes were evenly distributed where necessary. The time relationships of the vaccination and challenge procedures employed are illustrated below.



Collection and use of mouse sera. Fifteen days after the completion of vaccination sera were taken from mice for the purpose of demonstrating either agglutinin or protective antibody. The sera were separated from bloods collected according to the method of Evans and Perkins (1954). They were stored at +4°C and were used within 3 days. None was inactivated.

Each agglutination test was performed on a pool of equal volumes of 6 individual mouse sera. No assessment was made of the degree of accuracy to which this procedure indicated true average agglutinin titres. The agglutination tests were carried out using 0.4 ml. volumes of two-fold serum dilutions in the presence of equal volumes of a stock formolised suspension of strain No. 7157H diluted to the opacity of Brown's Tube 3.5. Titres were read after 2 hours incubation at 55°C.

In the case of sera to be examined for protective antibody content no attempt was made to pool precisely equal volumes from individual mice. The control serum was separated from the bulked bloods of 56 two-month old untreated mice and was sterilised by filtration. The two immune sera were obtained and prepared in a similar manner from 2 vaccinated groups of 36 mice each.

Passive immunity tests and non-specific protection. In the serum protection experiment dilutions of immune and control mouse sera were injected intraperitoneally in dose volumes of 0.5 ml. per mouse, 5 to 6 hours before challenge with Past. haemolytica in mucin.

In order to determine whether the methods used might produce a non-specific immunity in mice the following procedures were carried out 5 to 6 hours before challenge, (a) the intraperitoneal insertion and withdrawal of a hypodermic needle; (b) the intraperitoneal injection in sterile 0.5 ml. amounts of normal saline*, pH 6.75, veronal-saline buffer, 1/10 dilution of normal mouse serum in normal saline, respectively. All fluids introduced into the peritoneal cavity were first heated to approximately 37°C.

RESULTS

(a) Production of active immunity

Five groups of 58 mice each were vaccinated by the methods shown in Table 9 and a 6th group of the same size was left untreated for control purposes. All vaccines were administered intraperitoneally with the exception of the adjuvant vaccine which was injected subcutaneously on the inner aspect of the thigh.

Daily weighings of mice showed that injection of the formol-saline vaccine caused a loss of weight in 24 hours averaging 1.7 g.

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* This was prepared as a 0.85 per cent solution of sodium chloride in tap water.

per mouse. In the same period the injection of living vaccine resulted in an average loss of 0.8 g. per mouse while adjuvant vaccine merely produced a temporary suspension of weight increase. These disturbances to the mice were apparently rapidly overcome, and at the time of challenge each of the 6 groups of 58 showed an average mouse weight within the range of 25.6 g. and 26.2 g.

Six mice in each group were killed in order to collect serum for agglutination tests. The remaining 52 in each group were divided into 2 sub-groups of 26 mice each since 2 challenge doses were used. The results recorded in Table 9 show that each method of vaccination protected mice against challenge with Past. haemolytica in mucin and in some cases the protection was very strong. Comparison of the figures for agglutinin titres and deaths in mice receiving single doses of living or formol-saline vaccine with those for mice receiving adjuvant vaccine suggests a lack of correlation between agglutinin production and degree of protection.

(b) Demonstration of humoral protective antibody

Three groups of mice were used in the way already described to obtain sera for use in passive mouse protection tests. The first group had previously received one dose of formol-saline vaccine, the second two doses and the third group had not been vaccinated. Serum pools from each of the 3 groups were injected intraperitoneally into mice in saline dilutions of 1/2 and 1/20, and in dose volumes of 0.5 ml.

Subsequent challenge with a single dose level of organisms demonstrated conclusively that sera from both groups of vaccinated mice protected at the 1/2 dilution, and also that the serum from mice

TABLE 9

PRODUCTION OF ACTIVE IMMUNITY USING VARIOUS VACCINATION PROCEDURES

			Deaths in groups of 26 mice using 2 challenge levels		Total deaths	Agglutinin titres
			39×10^6 organisms	19.5×10^6 organisms		
Route and method of vaccination	I.P.	1 dose living vaccine	9	6	15/52	1/16
		2 doses living vaccine	1	0	1/52	1/1024
		1 dose formol-saline vaccine	3	2	5/52	1/64
		2 doses formol-saline vaccine	1	0	1/52	1/512
	S.C.	1 dose adjuvant vaccine	17	13	30/52	1/256
Unvaccinated controls			25	23	48/52	0

I.P. = intraperitoneal.

S.C. = subcutaneous.

Challenge doses injected intraperitoneally with mucin.

TABLE 10

DEMONSTRATION OF PROTECTIVE ANTIBODY IN SERA TAKEN FROM VACCINATED MICE

		Deaths produced by challenge in mice pretreated I.P. with doses of mouse sera	
		0.5 ml. 1/2 serum	0.5 ml. 1/20 serum
Sera obtained from mice previously treated as shown	1 dose formol-saline vaccine I.P.	0/12	3/12
	2 doses formol-saline vaccine I.P.	0/12	0/12
	Unvaccinated	10/20	11/20

I.P. = intraperitoneally.

All mice challenged intraperitoneally with 21×10^6 viable bacteria in mucin 5 to 6 hours after injection of serum.

which had received 2 doses of vaccine protected at the 1/20 dilution. The results of this experiment are shown in Table 10.

(c) Comparison of intraperitoneal and subcutaneous routes of vaccination

A single dose of formol-saline vaccine was injected intraperitoneally into mice of one group and subcutaneously in the dorsal region of mice of a second group. A third group did not receive vaccine.

Table 11 shows the results obtained by challenging these mice with 2 dose levels of Past. haemolytica. The test was read at the 18th hour following challenge, and again at 72 hours after which time no further deaths occurred. It is clear that while intraperitoneal vaccination gave strong protection from death, subcutaneous vaccination afforded little or none although it undoubtedly did increase the survival time. In addition, a pool of serum from six mice vaccinated intraperitoneally showed a higher agglutinin titre than did a similar pool taken from subcutaneously vaccinated mice.

(d) Comparison of intraperitoneal and intravenous routes of vaccination

The general design of this experiment was similar to that of the previous one. The results are set out in Table 12 and show that with regard to production of immunity and of agglutinin no significant difference between the two methods of vaccination could be detected.

Although no exact comparison with previous experiments is possible it is noticeable that the levels of protection and agglutinin produced by intraperitoneal vaccination in this experiment were somewhat lower than might have been expected. The type of agglutination was also unusual in that it was of a rather incomplete nature. This was the last experiment in which formol-saline vaccine was used and

TABLE 11

COMPARISON OF INTRAPERITONEAL AND SUBCUTANEOUS ROUTES OF VACCINATION

		Vaccinated I.P.		Vaccinated S.C.		Unvaccinated controls	
Hours after challenge		18	72	18	72	18	72
Deaths in groups of 19 mice challenged as shown	40×10^6 organisms	1	7	7	17	15	19
	20×10^6 organisms	0	2	8	17	14	19
Agglutinin titres		1/64		1/8		0	

I.P. = intraperitoneally.

S.C. = subcutaneously.

Challenge doses injected intraperitoneally with mucin.

TABLE 12

COMPARISON OF INTRAPERITONEAL AND INTRAVENOUS ROUTES OF VACCINATION

	Deaths in groups of 22 mice using 2 challenge levels		Agglutinin titres
	34×10^6	17×10^6	
Vaccinated I.P.	15	6	1/32
Vaccinated I.V.	18	7	1/32
Unvaccinated controls	20	20	0

I.P. = intraperitoneally.

I.V. = intravenously.

Challenge doses injected intraperitoneally with mucin.

by this time the vaccine was almost four months old.

(e) Immunity not related to antibody

In carrying out studies on immunity a strong suggestion was obtained that injection of certain fluids devoid of antibody into the peritoneal cavities of mice was capable of increasing resistance to intraperitoneal challenge with Past. haemolytica 5 to 6 hours later. In regard to the passive immunity test recorded in Table 10 it will be seen that approximately 50 per cent mortality was obtained in control mice which had received normal mouse serum several hours before challenge with 21×10^6 organisms. However the deaths among controls in those experiments recorded in Tables 9, 11 and 12 indicate that smaller doses of organisms may be capable of producing considerably higher mortality in mice not pretreated with normal serum.

To investigate the matter further an experiment was performed in which 3 groups of mice received intraperitoneal injections of 1/10 mouse serum, normal saline or veronal-saline buffer respectively 5 to 6 hours before intraperitoneal challenge with Past. haemolytica in mucin. At the same time mice of a fourth group were stabbed intraperitoneally with a hypodermic needle. A fifth group consisted of untreated control mice. These 5 groups were divided into sub-groups to allow for challenge with 4 dose levels of organisms. The results are shown in Table 13.

Injection of each of the three fluids resulted in increased resistance to challenge, but these protective effects were apparent only in mice which received the lower doses of organisms and were obliterated by the higher challenge levels. The trauma produced by a

TABLE 13

PRODUCTION OF NON-SPECIFIC RESISTANCE BY VARIOUS TREATMENTS

		Deaths in groups of 10 mice challenged at 4 levels				Total deaths produced by 2 smaller challenge doses
		27×10^6 organisms	13.5×10^6 organisms	6.75×10^6 organisms	3.3×10^6 organisms	
Mice pretreated as shown, 5 to 6 hours before challenge	0.5 ml. 1/10 mouse serum I.P.	10	7	4	2	6/20
	0.5 ml. normal saline I.P.	9	6	0	0	0/20
	0.5 ml. veronal-NaCl buffer I.P.	9	9	5	2	7/20
	Needle trauma alone	10	10	8	3	11/20
Untreated controls		9	10	9	8	17/20

I.P. = intraperitoneally.

Challenge doses injected intraperitoneally with mucin.

needle may also have resulted in some increased resistance but a further experiment would have been required to decide this matter conclusively.

PART 3

STUDIES RELATED TO THE PATHOGENICITY OF PAST. HAEMOLYTICA FOR SHEEP

A. INTRATRACHEAL INOCULATION OF SHEEP

MATERIALS AND METHODS

The experiments to be described were performed on different occasions over a period of two years.

Sheep. The ages of animals inoculated varied from approximately 7 months to 1 year.

Inocula. Past. haemolytica strains Nos. 107, 108, 128 and 140 were used and their histories are given in Appendix I. They were all derived from cases of enzootic pneumonia and were injected either in the form of suspensions in 1 per cent casein hydrolysate solution of primary blood agar cultures or in diseased lung suspensions. Each lung suspension was prepared by disrupting 5 gm. of tissue in 10 ml. of 1 per cent casein hydrolysate solution in a Griffith tube. After light centrifugation the cloudy, red supernate was pipetted off and used for inoculation. Viable counts of Past. haemolytica in both tissue and culture suspensions were made immediately before injection. The average of 3 replicate counts was in each case taken to be the true value. Certain inocula incorporated mucin suspension prepared as described earlier.

Intratracheal Inoculations. Sheep were held in the upright position while inoculations were made and for two minutes afterwards. Material

was injected very slowly into the trachea as near the chest entrance as possible. In certain cases inoculations were made under deep anaesthesia.

Observations on Inoculated Sheep. Sheep were examined clinically every 24 hours for a period of 10 days after inoculation.

RESULTS

Sheep were inoculated with various strains of the organism as shown below. Throughout the period of observation none of the animals inoculated showed elevated temperatures or any other sign of disease.

Strain 107. Doses of 5 ml. of plate culture suspension each containing 45,000 x 10^6 viable bacteria were administered to 2 one-year-old sheep.

Strain 108. Injections were made into 1-year-old sheep under deep anaesthesia. Each of 2 sheep received 2 ml. of lung tissue suspension containing 1100 x 10^6 viable organisms. Two further sheep were given doses of 800 x 10^6 organisms in the form of 2 ml. volumes of plate culture suspension.

Strain 128. The inoculum consisted of plate culture suspension mixed with an equal volume of mucin suspension. It contained 4000 x 10^6 viable organisms per ml. Three 7-month-old lambs received doses of 1 ml., 3 ml. and 5 ml. respectively.

Strain 140. Injections were performed under deep anaesthesia. A 1-year-old sheep received 2 ml. of lung suspension containing 12 x 10^6

living bacteria. A dose of 2 ml. of plate culture suspension containing 7000 x 10^6 organisms was inoculated into a second animal of the same age.

These negative results are similar to those obtained by the majority of previous workers.

B. VIRULENCE FOR SHEEP BY INTRAVENOUS INOCULATION

MATERIALS AND METHODS

Strain. 7157H, freeze-dried after a single mouse passage.

Sheep. Eight adult sheep were used. Each showed agglutinins to strain 7157H but in no case did the titre exceed 1/16.

Inocula. These were prepared from a 24 hour infusion broth culture containing 300 x 10^6 viable organisms per millilitre. Doses consisted of 5.0 ml. volumes of either undiluted culture or 1/100 culture injected intravenously.

RESULTS

Four sheep which received undiluted broth culture died. Four sheep which received 1/100 dilution of broth culture survived without showing symptoms. Of the animals given lethal doses 3 succumbed within 18 hours of inoculation. The fourth died after 3 days during which time it showed symptoms including elevated temperature, extreme listlessness, clear nasal discharge and diarrhoea; in addition blood smears and cultures made on the day preceding death were positive for Past. haemolytica. The post-mortem appearance of those sheep which died was characterised by marked congestion, oedema and haemorrhage,

and in each case Past. haemolytica was cultured from lung, liver, spleen, kidney and heart blood.

C. BACTERIOLOGY OF 3 FATAL INFECTIONS PRODUCED BY INTRAVENOUS INOCULATION

MATERIALS AND METHODS

Strain. 7157H, freeze-dried after a single mouse passage.

Sheep. Three 2-year-old sheep were used, their weights ranging from 94 to 102 lbs. Agglutination tests on sera collected shortly before inoculation showed in each case a titre against strain 7157H not exceeding 1/16. The infection of these 3 sheep constituted separate experiments performed on 3 different occasions within a period of 1 month.

Inocula. In the light of results of the previous experiment the dose per sheep was fixed at 5.0 ml. of undiluted 22 hour infusion broth culture injected intravenously. Viable counts of inocula performed in triplicate immediately before injection showed that the 3 sheep received the following doses of living bacteria: Sheep 1, 4250×10^6. Sheep 2, 550×10^6. Sheep 3, 4500×10^6.

Bacterial Counts on Blood. These were performed at various intervals between the time of inoculation and death. The method used was to withdraw aseptically 4.0 ml. of blood from the jugular vein and to transfer immediately to a bottle containing 1.0 ml. of 3.75% sterile trisodium citrate solution to prevent clotting. Within a few minutes of bleeding 3 series of 10-fold dilutions from 10^{-1} to

10^{-6} were prepared in 1% casein hydrolysate solution from the citrated blood sample and used to perform viable counts in triplicate. In addition 1/50th ml. of undiluted blood-citrate mixture delivered by means of a dropping pipette was used to inoculate 10 ml. of broth in order to detect the presence of smaller numbers of bacteria. The procedure of citration was known to have no adverse effect on the viability of Past. haemolytica. This had already been shown by an experiment in which survival in heavily citrated blood over a period of 3 hours proved to be as satisfactory as survival in 1% casein hydrolysate solution. Numbers of organisms in the bloodstream at the moment of inoculation were calculated from the numbers of bacteria introduced on the assumption that the blood volume of a sheep is equivalent to 5% of its body weight.

Bacterial Counts on Tissues. Immediately after death a post-mortem was carried out and portions of various tissues were transferred to the refrigerator. Within a few hours thereafter 5 gm. amounts of each organ were removed aseptically. This was done by immersing a large piece of tissue for 5 to 10 seconds in boiling water and subsequently dissecting out 5 gm. from the central unheated part using sterile instruments. Bone marrow was obtained by dissection from the seared, cut surface of a long bone which had been sawn through near the centre of the shaft. In each case the weighed tissue was chopped finely with scissors and added to 20 ml. of sterile 1% casein hydrolysate solution in a Griffith tube in which it was then macerated. Each resulting suspension was considered to be a 1/5 tissue suspension and was used to prepare series of

10-fold dilutions for triplicate viable counts. Fluids such as pericardial exudate were collected aseptically and dilution series for counting were prepared directly from them.

RESULTS

Sheep Nos. 1, 2 and 3 died 15½, 21 and 23 hours respectively after inoculation. Temperatures were taken at frequent intervals and as may be seen in Figure 2 the 3 sheep experienced similar diphasic temperature reactions. The first peak occurred at the 5th or 6th hour after injection and this was followed by a marked decline terminating between the 10th and 13th hour. Thereafter the temperature rose fairly steadily until the time of death.

Sheep No. 1 and 2, but not No. 3, showed mild symptoms at the 3rd or 4th hour after inoculation. These consisted of unwillingness to move and laboured breathing with slight grunting on expiration, but after a further period of 2 or 3 hours they had disappeared. At the 10th hour the outward appearance of all 3 sheep was quite normal. Within a short period however signs of illness began to appear and these increased in severity until death. This final train of symptoms consisted of listlessness, diarrhoea, weakness followed by prostration, frothing at the mouth and nose, and severe dyspnoea with grunting on expiration.

Viable counts of organisms in the blood of the 3 sheep gave the results shown in Table 14. Within 5 minutes of inoculation many organisms had been removed from the circulation so that either none or very few could be demonstrated. Throughout the course of the infection blood viable counts tended to be low or in many cases blood

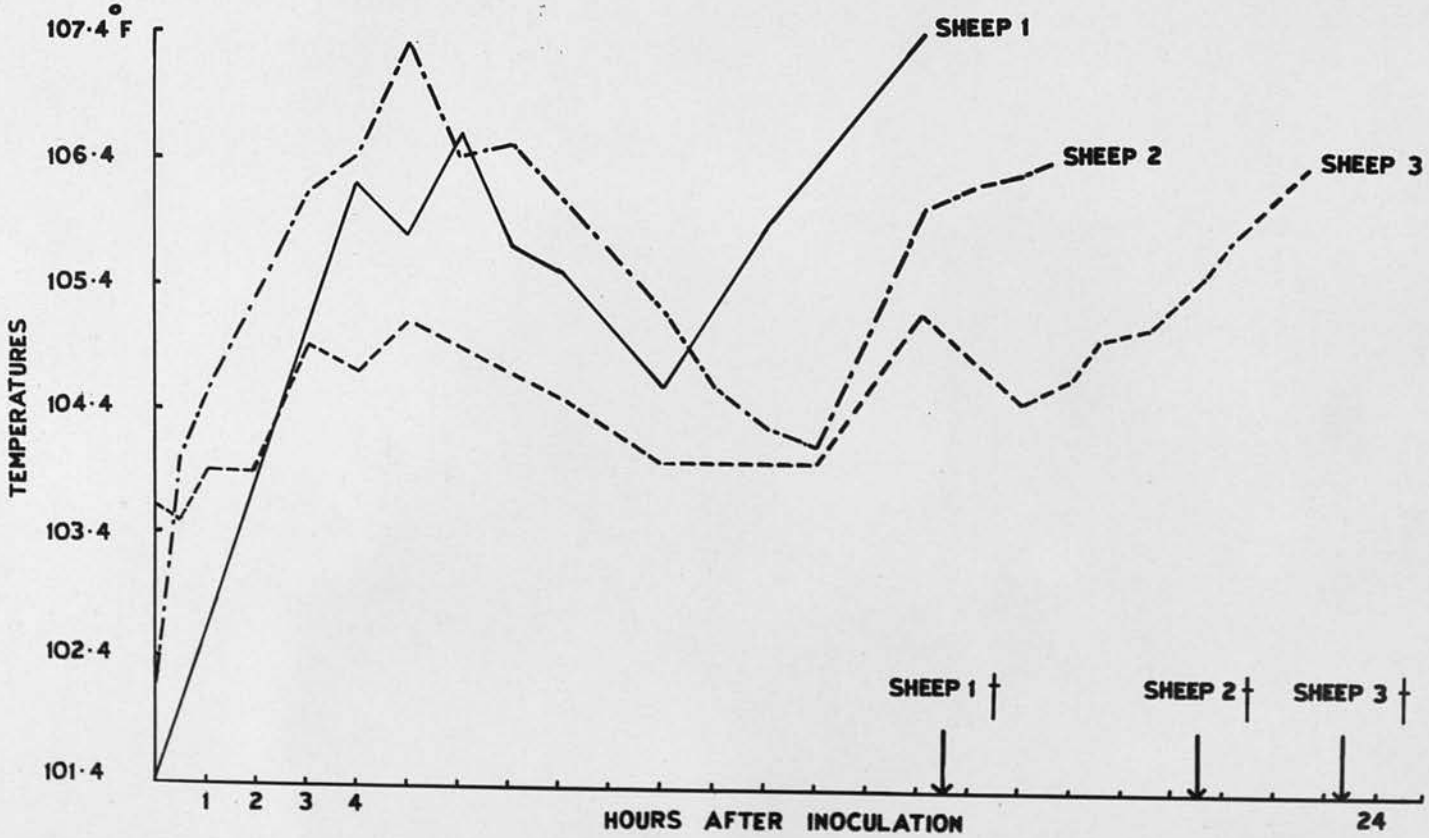

FIG. 2. TEMPERATURE REACTIONS IN 3 SHEEP INOCULATED INTRAVENOUSLY WITH LETHAL DOSES OF STRAIN 7157H

TABLE 14

VIABLE BACTERIA IN BLOOD OF 3 SHEEP INOCULATED INTRAVENOUSLY WITH STRAIN 7157H

Time after inoculation	Viable counts of bacteria in blood of 3 sheep		
	Sheep 1	Sheep 2	Sheep 3
0 min.	2000	200	2000
5 min.	+	0	0
30 min.	5	0	0
1 hr.	3	0	0
2 hr.	1354	0	0
3 hr.	156	0	0
4 hr.	167	0	0
5 hr.	1854	0	0
6 hr.	129	0	+
7 hr.	1417	+	+
8 hr.	2750	+	N
10 hr.	604	0	118
12 hr.	148	+	N
13 hr.	N	N	+
15 hr.	2146	+	+
15 hr. 30 min.	died	N	N
16 hr.	15000	N	N
17 hr.	....	+	2
19 hr.	....	N	3
20 hr. 40 min.	....	died	4
22 hr. 40 min.	....	....	16
23 hr. 20 min.	....	....	died 94
23 hr. 30 min.	....	....	394

Viable counts given in thousands per ml. of blood.
+ = bacteria present but not exceeding 625 per ml.
0 = 10 ml. of broth inoculated with 1/50th ml. of undiluted blood citrate mixture gave no growth.
N = not done.

cultures were negative. There was however a tendency towards increasing counts in blood samples withdrawn shortly before and after death. In the instance of sheep No. 3 in which the penultimate blood sample was taken at the precise moment of death there clearly occurred an increase in numbers of bacteria in jugular blood in the 10 minutes immediately following death.

The macroscopic and histological post-mortem findings were as described under pathology of experimental Past. haemolytica septicaemia by Stamp et al., except that no bacterial emboli were seen in lung tissue. They were however particularly obvious in spleen.

Table 15 shows the numbers of viable organisms per gram of various tissues removed from the 3 sheep at death. In each case counts of bacteria in spleen were vastly greater than those in any other tissue examined.

TABLE 15

VIABLE BACTERIA IN TISSUES AND FLUIDS AT DEATH.
3 SHEEP INOCULATED INTRAVENOUSLY WITH STRAIN 7157H

Tissues and fluids examined	Viable counts in tissues and fluids of 3 sheep		
	Sheep 1	Sheep 2	Sheep 3
Lung	19.2	5.5	26.2
Liver	6.3	10.2	21.2
Spleen	3082.0	975.0	800.0
Kidney	9.2	1.4	13.5
Bone marrow	1.1	N	N
Pericardial fluid	0.0005	0.001	N
Pleural fluid	N	1.6	N
Tracheal fluid	0.06	6.0	N

Viable counts given in millions per gm. of solid tissues and per ml. of fluids.

N = not done.

D. INOCULATION OF A SHEEP PROTECTED WITH STREPTOMYCIN

The purpose of this experiment was to investigate the possible toxic effects of an intravenous inoculum of 5 ml. of 22 hour broth culture in a sheep given massive doses of streptomycin to suppress bacterial multiplication. The dose of bacteria injected was known from the foregoing experiments to be lethal for unprotected sheep.

MATERIALS AND METHODS

Strain. No. 7157H, freeze-dried after a single mouse passage.

Sheep. A 2 year old sheep weighing 110 lbs. A serum sample collected the day before inoculation showed an agglutinin titre of 1/16 to strain 7157H.

Inoculum. 5 ml. of 22 hour infusion broth culture injected intravenously. The average of 5 replicate viable counts showed that this inoculum contained 1515×10^6 living bacteria.

Streptomycin injections. These were given in the form of streptomycin calcium chloride. 2 gm. were given subcutaneously and a further 1 gm. intravenously 45 mins. and 1 min. respectively before infection. After infection 1 gm. doses were given at the 2nd and 4th hours intramuscularly, and at the 7th and 13th hours subcutaneously.

Blood cultures. 1 ml. of blood was withdrawn aseptically from the jugular vein and inoculated into 10 ml. of broth. Before this mixture was incubated it was used to inoculate 2 further broths and 1 blood agar plate, the inoculum in each case consisting of a single loopful. In this way any excessive amounts of streptomycin present in the blood would have been greatly diluted.

RESULTS

45 mins. before inoculation the sheep was extremely alert and lively, and its temperature was 104°F. 1 hour after inoculation the temperature was 105.4° and after a further hour had risen to 106°. For the next 5 hours the temperature fluctuated between 105.8° and 106.1° but at the 10th hour after inoculation it had fallen to 104° and remained normal for the next 3 days after which time the experiment was discontinued. Between the 1st and 5th hours after inoculation the sheep showed definite signs of illness consisting of unwillingness to stand and grunting on expiration, particularly when handled. Recovery was rapid however and at the 7th hour the animal appeared normal once more. Blood cultures made at the 7th and 13th hours after inoculation were negative.

E. TOXIC EFFECT OF HEAT KILLED BROTH CULTURE INJECTED INTRAVENOUSLY

MATERIALS AND METHODS

Strain. 7157H, freeze-dried after a single mouse passage.

Sheep. 2 adult sheep were used. The injection of the 2 sheep constituted separate experiments performed within a few days of each other.

Inocula. These consisted of 22 hour infusion broth cultures sterilised by heating at 56°C for 15 minutes and injected intravenously. Sheep 1 received 10 ml. Sheep 2 received 50 ml. initially followed 3 hours later by a second dose of 30 ml.

RESULTS

Both sheep were examined clinically at various periods after inoculation and the results are shown below.

Sheep 1

30 minutes: The temperature had risen from 102.1°F to 105.2°F. The animal was lively but on handling it began to tremble severely, groups of muscles over the neck, shoulder and flank being involved.

1 hour: Temperature 107.3°. Trembling had ceased and the sheep appeared normal.

2 hours: Temperature 106.6°. No other symptoms were observed.

3 hours: Temperature 107.7°. Extreme hyperexcitability was noticed and trembling had recommenced. Signs of diarrhoea were present.

4 hours: Temperature 108.5°. Symptoms of trembling and diarrhoea were still present but to a slight degree.

24 hours: Temperature 104.4°. The animal appeared normal. No further symptoms or rises in temperature were observed.

Sheep 2

30 minutes: The temperature had risen from 102° to 104°. The sheep showed marked respiratory distress with gurgling noises in the throat and almost explosive expirations. It refused to move unless deliberately chased whereupon the respiratory symptoms became even more marked.

1 hour: Temperature 104.6°. The symptoms were as recorded above and in addition diarrhoea had commenced.

1½ hours: Temperature 104.7°. The sheep was standing stock still in a pool of liquid faeces. Respiratory distress had become even more acute but the animal could still trot quickly if chased. Slight ruminal tympany was observed.

2½ hours: Temperature 103.8°. Tympany had become more exaggerated and the sheep was incapable of moving quickly. Breathing seemed slightly easier.

3½ hours: Temperature 103.4°. The sheep was recumbent and refused to rise.

5½ hours: Temperature 101°. The animal was still recumbent and was hypersensitive to touch over the abdominal region.

9 hours: Temperature 102.2°. Diarrhoea had ceased and respiratory distress was much less acute.

11 hours: Temperature 102.2°. The sheep was standing and appeared to be more comfortable. Tympany was still present but dysponoea was slight.

21 hours: The animal was found dead.

Post Mortem Examination of Sheep 2

Multiple widespread subcutaneous haemorrhages were present. These were particularly marked in the neck region from the lower jaw to the chest entrance, over the thorax and in the groin. The thorax contained approximately 1500 ml. of a clear, straw-coloured serous fluid with a few small jelly-like clots. The mediastinal tissues were thickened by straw-coloured jelly-like oedema. The external surface of the trachea was covered with small haemorrhages. Internally the trachea was inflamed and contained a considerable

amount of very frothy fluid. The lungs were of a light purple hue and felt rather emphysematous. The right apical lobe was of firmer consistence than normal but still considerably aerated. It and the right cardiac lobe showed several sunken linear areas of collapse. A cut surface of both diaphragmatic lobes showed the lobular pattern outlined by perilobular congestion. The pericardial sac contained about 75 ml. of a clear serous fluid. The heart muscle was pale and the wall of the right ventricle extremely flabby. Both auricles, particularly the right auricle, showed marked external petechiation. The right ventricular endocardium was covered with ecchymotic haemorrhages. The left ventricle was normal. The abdominal cavity contained a considerable quantity of blood-tinged serous fluid. The rumen was distended with gas and its external surface showed a few small haemorrhages. The abomasal mucosa was normal. The small intestine throughout its length was inflamed and showed many scattered tiny petechiae on the mucosal surface. In one section there was an appearance of catarrhal enteritis with cream-like intestinal contents. The liver possessed a yellow toxic appearance and a cut surface showed small dark haemorrhagic areas scattered somewhat sparsely throughout the substance and apparently associated with the larger blood vessels. The renal capsules stripped easily. The kidneys showed dark red cortices in which the medullary rays stood out as in an early stage of pulpy kidney disease. The spleen was flabby and showed a few areas of subcapsular haemorrhage.

Histological examination of tissues revealed widespread congestion. Lung tissue showed in addition emphysema, alveolar

and interlobular oedema and small areas of collapse. Sections of kidneys showed cortical necrosis of convoluted tubules.

F. PYROGENIC EFFECT OF BROTH CULTURE FILTRATES INJECTED INTRAVENOUSLY

The two previous experiments provided information on toxicity of whole broth cultures both in the living state and after sterilisation by heat. A description is now given of an experiment designed to investigate the possible toxic effects of broth cultures freed from bacterial cells by filtration.

MATERIALS AND METHODS

Strains. The strains used were Nos. 7157H, 32, 34 and 36 and further details of these may be found in Appendix I. No. 7157H was freeze-dried after a single mouse passage while the other 3 strains had been maintained for varying periods in culture.

Sheep. 15 adult sheep were used.

Inocula. These consisted of sterile filtrates of infusion broth cultures of varying ages. Bacteria were removed from each culture by filtration through a pad composed of 3 membrane filters* interleaved with filter paper. Sheep were injected intravenously with the various inocula shown below. Inocula for sheep Nos. 1 to 10 inclusive were prepared using strain 7157H.

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* Manufactured by Oxoid Ltd., London.

Sheep 1: 50 ml. of 6 hr. c.f.[**]

Sheep 2: 20 ml. of 22 hr. c.f.

Sheep 3: 20 ml. of 22 hr. c.f. stored for 20 hrs. at +4°C.

Sheep 4: 10 ml. of 22 hr. c.f. followed 3 hrs. later by a further 15 ml.

Sheep 5: 475 ml. of 22 hr. c.f.

Sheep 6: 20 ml. of 22 hr. c.f. heated at 56°C for 1 hr.

Sheep 7: 20 ml. of 22 hr. c.f. after storage for 20 hrs. at +4°C in the presence of 0.5% formalin.

Sheep 8: 15 ml. of 24 hr. c.f. 16 days previously this animal had received 50 ml. of 6 hr. c.f. and had shown a temperature reaction.

Sheep 9: 30 ml. of 7 day c.f.

Sheep 10: 12 ml. of 16 day c.f.

Sheep 11: 20 ml. of 18 hr. c.f. prepared using strain 32.

Sheep 12: 20 ml. of 18 hr. c.f. prepared using strain 34.

Sheep 13: 20 ml. of 18 hr. c.f. prepared using strain 36.

Sheep 14 (control): 10 ml. of uninoculated sterile infusion broth followed 3 hrs. later by a further 15 ml.

Sheep 15 (control): 500 ml. of uninoculated sterile infusion broth.

RESULTS

Temperatures of all 15 sheep were taken immediately before inoculation and at various intervals afterwards. These are recorded in Table 16. Sheep Nos. 1 to 13 inclusive showed well marked thermal reactions which commenced within a short period after

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[**] c.f. = culture filtrate.

TABLE 16

PYROGENIC EFFECT IN SHEEP OF PAST. HAEMOLYTICA BROTH CULTURE FILTRATES INJECTED INTRAVENOUSLY

Hours after inoculation	Temperature reactions of 15 sheep														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
0	103.2	102.4	103.6	103.3	102.6	103.0	102.5	101.4	100.8	101.5	102.2	102.6	101.8	103.0	102.2
0.5	104.3	104.4	N	N	105.6	104.3	N	N	102.9	N	N	N	N	N	102.9
1.0	106.4	N	N	104.1	105.1	N	N	N	103.8	N	103.4	104.3	103.8	103.6	102.8
1.5	N	106.5	105.6	105.5	N	105.7	104.7	N	103.8	N	103.7	105.0	104.6	103.9	N
2.0	N	N	N	N	104.3	N	N	105.5	N	104.2	N	N	N	N	102.5
2.5	106.2	N	N	105.5	N	N	N	N	104.5	N	N	N	N	103.9	N
3.0	N	106.2	N	N	106.1	106.8	N	106.7	N	106.2	105.1	105.6	106.2	N	102.9
3.5	107.5	N	104.3	N	N	N	106.2	N	N	N	N	N	N	N	N
4.0	107.9	N	N	106.7	106.9	N	N	106.2	106.0	106.3	106.7	106.5	106.9	103.4	104.0
4.5	N	N	N	107.1	N	N	N	N	106.0	N	N	N	N	103.3	N
5.0	N	N	104.6	107.5	106.0	N	104.9	105.7	N	106.2	N	N	N	103.3	104.3
5.5	106.7	N	N	106.7	N	N	N	N	105.7	N	106.7	105.9	106.2	103.3	N
6.0	N	104.0	N	106.6	N	103.7	N	N	N	N	N	N	N	103.2	N
6.5	N	N	N	N	104.9	N	N	N	104.6	N	N	N	N	N	104.2
7.0	N	N	103.2	106.0	N	N	103.7	N	N	N	N	N	N	103.2	N
24.0	103.1	103.4	103.1	104.1	104.1	102.1	102.5	102.2	101.7	103.8	103.4	103.4	103.3	103.6	103.0

Sheep nos. 1 to 13 received broth culture filtrates as described in text.
Control sheep nos. 14 and 15 received uninoculated, sterile broth as described in text.
N = not done.

inoculation and usually reached maximum levels between the 3rd and 5th hours. In all cases temperatures were normal on the following day. This pyrogenic effect of broth culture filtrates occurred using each of 4 different strains of Past. haemolytica and appeared to be independent of the age of the culture. It was not abolished by treatment of the filtrate with heat or formalin. The sheep which had received culture filtrate 16 days previously reacted again to a second injection. With the exception of sheep No. 5 none showed any definite symptoms other than high temperature. 30 mins. after inoculation sheep No. 5 showed symptoms of listlessness and laboured breathing with grunting on expiration. After a further 2 hours had elapsed it appeared normal once more. Control sheep Nos. 14 and 15 showed neither increased temperatures nor any sign of illness after receiving injections of uninoculated sterile broth.

G. BACTERIOLOGY OF A FIELD CASE OF SEPTICAEMIA

An outbreak of Past. haemolytica septicaemia similar to those described by Stamp et al. (1955) produced 14 deaths in a batch of 250 lambs over a period of two weeks. The animals concerned were 7 months old. One infected lamb was brought to the laboratory in a moribund state so that the distribution of viable organisms in various tissues could be determined immediately after death.

MATERIALS AND METHODS

Bacterial Counts on Tissues. These were performed on lung, liver, spleen, kidney and mesenteric lymph gland by the method already described.

RESULTS

The lamb showed acute respiratory distress, diarrhoea and a temperature of 108°F one hour before death. Post-mortem examination revealed widespread subcutaneous haemorrhages up to 2 cm. in diameter. The tracheal mucous membrane was intensely congested. The lungs showed deep purple coloration, oedema and many sub-pleural haemorrhages. Sub-epicardial haemorrhages were profuse in the region of the coronary vessels and sub-endocardial ecchymoses were present in the left ventricle. The abomasal mucosa was covered with petechiae and the intestines showed congestion.

Counts of viable Past. haemolytica in various tissues gave the following results.

Lung	450×10^6 per gm.
Liver	550×10^6 " "
Spleen	65×10^6 " "
Kidney	0.1×10^6 " "
Mesenteric lymph gland	0.07×10^6 " "

PART 4

A. TWO TYPES OF PAST. HAEMOLYTICA AND THEIR ASSOCIATION WITH DIFFERENT FORMS OF DISEASE IN SHEEP

In the course of studies on Past. haemolytica isolated from cases of pneumonia and septicaemia it proved possible to place each of the strains examined into one of two main groups which, though closely similar, were distinguishable by certain in vitro characteristics. These two groups have been designated A and T. For the sake of clarity the reference number of each strain used in the following series of experiments is preceded by the letter A or T to indicate its type as determined by the methods which are now to be described.

MATERIALS AND METHODS

Strains of Past. haemolytica. Of the strains used Nos. A-10 and A-36 were isolated from the nasal passages of apparently healthy sheep. The remainder were obtained either from cases of pneumonia in lambs and sheep of varying ages or from cases of septicaemia in lambs between 6 and 12 months of age. Further details of all strains may be obtained by referring to Appendix I.

Fermentation reactions. These were carried out in the medium recommended by Bosworth and Lovell (1944). This consisted of peptone water containing 10% infusion broth, 7.5% brom-thymol-blue and 1% of the fermentable substance. The pH was adjusted to 7.0-7.3. With regard to pH considerable adjustment was necessary in media containing arabinose, xylose and to a lesser degree in those containing galactose, fructose, maltose and mannose since these

sugars showed variable tendencies to break down and produce acidity on tyndallisation. Each strain was tested in the presence of 24 fermentable substances and also in a control tube containing the basic medium only. Tubes were inoculated with one drop of a 5 hour broth culture delivered by means of a finely drawn Pasteur pipette and only those which became completely yellow after incubation were regarded as positive.

Examination of broth cultures. The viable counts, opacities and pH values of broth cultures incubated at 37°C were recorded at various intervals after inoculation.

Viable counts were performed by the method described in Appendix II, the average of 3 replicate counts being taken as the true value.

Opacities were measured in standard screw-capped glass test-tubes by means of an EEL nephelometer. On each occasion that readings were made the instrument was first standardised using a suspension of barium sulphate having the opacity of No. 0.5 on McFarland's Scale. This was prepared by adding 1% barium chloride solution to a 1% solution of sulphuric acid in the proportion of 995 volumes to 5 volumes (McFarland, 1907). Thus the nephelometer readings could without difficulty be converted to McFarland's Scale. No allowance was made for the colour of nutrient broth since the error due to this was found to be negligible. Before measuring opacities great care was taken to ensure that all suspensions were thoroughly shaken and that no air bubbles were present.

Hydrogen ion concentrations were measured using a direct reading pH meter and microelectrode.

Sensitivity to antibiotics. A preliminary test to investigate the sensitivity of a number of strains to various antibiotics was carried out by means of the absorbent paper disc technique (Morley, 1945). "Multodisks"* containing 10 mcg, 10 mcg, 5 mcg, 10 mcg, 10 mcg and 1.5 units respectively of chloromycetin erythromycin, oleandomycin, streptomycin, tetracycline and penicillin were used. Five per cent sheep blood agar plates were flooded with 1/10 dilutions of 5 hour infusion broth cultures of the various strains investigated. The excess moisture was removed and the plates allowed to dry. Multodisk papers were then placed on the surface of each culture. The tests were read after 18 hours incubation by measuring the distance in millimetres between the edge of each disc and the circumference of the corresponding zone of inhibition.

Sensitivity to penicillin was further investigated by the broth dilution method. Solutions in infusion broth of the sodium salt of crystalline penicillin G containing 2.5, 1.25, 0.625, 0.3125 and 0.1562 units per ml. were prepared. These were dispensed aseptically into sterile test-tubes in 1 ml. volumes. Inocula consisted of 1 ml. amounts of 6 hour infusion broth cultures diluted 10^{-6}. In this way each strain was tested in the presence of final concentrations of 1.25, 0.625, 0.3125, 0.1562 and 0.0781 units of penicillin per ml. Tests were read after 18 hours incubation by visual examination for the presence or absence of growth. In addition a loopful from each tube was plated out and incubated to see if viable bacteria were present.

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* Manufactured by Oxoid Division, Oxo Ltd.

RESULTS

(a) Colonial Morphology of A and T Strains

On the basis of colonial appearance each one of a considerable number of strains of Past. haemolytica was classified as type A or type T. Twenty-four hour colonies of T strains on 5% sheep blood agar measured up to 2mm. in diameter and when viewed by transmitted light they were seen to possess large dark brown centres, the colour fading markedly towards the peripheries (Figure 3). Colonies of A strains tended to be rather smaller and when viewed by transmitted light they showed an even lightish grey colour (Figure 4). A colonies sometimes showed a small clearly demarcated central thickening. When smears were made in distilled water from smooth T colonies the bacteria generally showed a strong tendency to become arranged in the lace-like pattern depicted in Figure 5. Smears made in saline showed even dispersal of organisms. Smears made in distilled water from A colonies usually showed even dispersal of bacteria and a number of weakly staining "ghost forms".

(b) Behaviour of A and T Strains in Fermentation Media

The results of tests on a total of 28 strains are given in Table 17. It will be seen that all T strains gave a positive reaction in trehalose fermentation medium after 2 days incubation while the reaction in arabinose medium was negative up to the 10th day. A strains gave a positive reaction in arabinose medium by the 7th day of incubation but remained negative in trehalose up to the 10th day. At the 14th day of incubation reactions in these 2 sugar media no longer completely differentiated A and T strains. In addition the fermentative capacity of A strains for starch, glycogen and glycerol

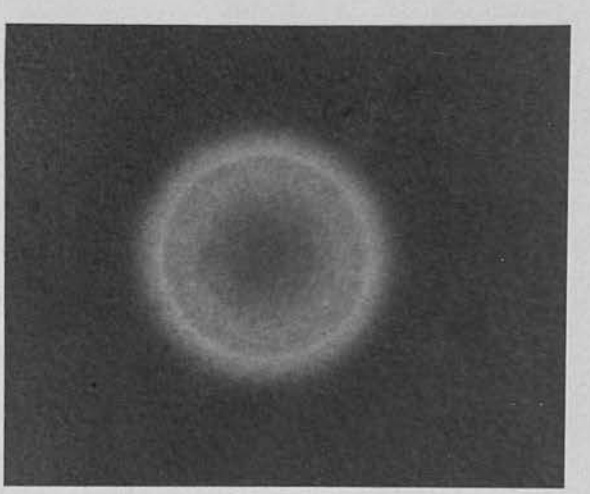


Fig. 3. Type T colony viewed by transmitted light after 24 hours' growth on blood agar. (X 15)

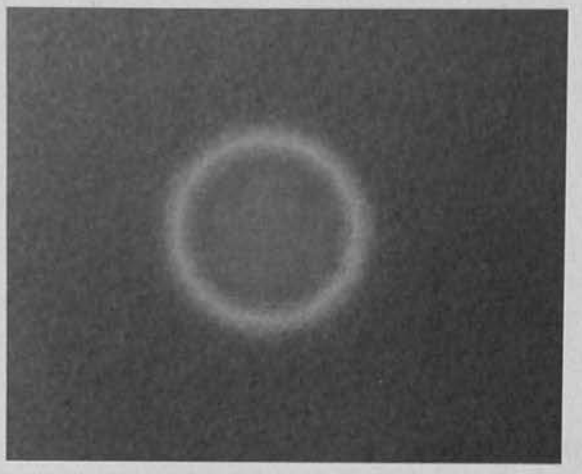


Fig. 4. Type A colony viewed by transmitted light after 24 hours' growth on blood agar. (X 15)

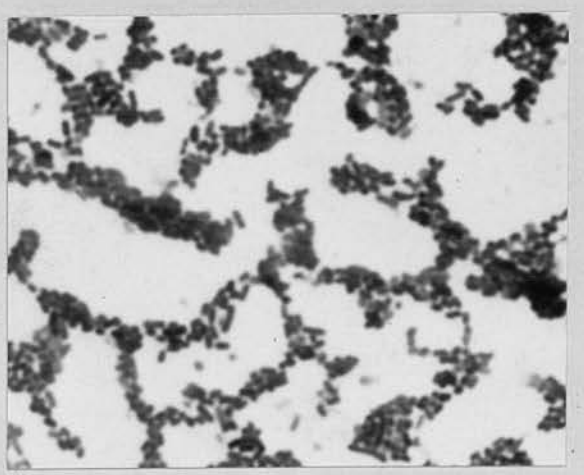


Fig. 5. Smear of T strain made in distilled water. (X 750)

TABLE 17

FERMENTATION REACTIONS OF A AND T STRAINS

	Positive fermentation reactions of 14 A strains and 14 T strains							
Period of incubation	2 days		7 days		10 days		14 days	
Type of strain	A	T	A	T	A	T	A	T
Arabinose	8	0	14	0	14	0	14	4
Xylose	13	11	14	14	14	14	14	14
Rhamnose	0	0	4	0	6	0	6	1
Glucose	14	13	14	14	14	14	14	14
Fructose	14	14	14	14	14	14	14	14
Mannose	0	12	14	14	14	14	14	14
Galactose	9	6	14	11	14	11	14	11
Sucrose	14	11	14	14	14	14	14	14
Maltose	14	14	14	14	14	14	14	14
Lactose	3	0	3	0	3	0	3	0
Trehalose	0	14	0	14	0	14	3	14
Raffinose	9	2	14	6	14	7	14	9
Starch	0	0	14	1	14	1	14	5
Inulin	0	0	0	0	0	0	0	0
Dextrin	14	14	14	14	14	14	14	14
Glycogen	0	0	4	0	11	0	11	0
Glycerol	1	0	7	3	11	3	11	4
Erythritol	0	0	0	0	0	0	0	0
Adonitol	0	0	0	0	0	0	0	0
Mannitol	13	12	14	14	14	14	14	14
Dulcitol	0	0	0	0	0	0	0	0
Sorbitol	14	14	14	14	14	14	14	14
Salicin	0	9	0	10	0	10	2	11
Inositol	0	0	12	10	12	11	12	13
Control medium	0	0	0	0	0	0	0	0

Only completely yellow tubes considered as positive.

Control medium contained no added fermentable substance.

tended to be greater than that of T strains. With mannose and salicin this situation was reversed although in the case of mannose the difference was merely one of speed of fermentation.

In a subsequent experiment using 15 A strains and 15 T strains viability in trehalose, arabinose and mannose media after 10 days incubation was investigated. This was done by plating out a loopful from each tube. All of the A strains survived over this period in the three media. All T strains survived in arabinose medium but none did so in the presence of trehalose or mannose.

(c) Observations on Broth Cultures of A and T Strains

In a preliminary experiment the growth curves in terms of viable organisms of two strains, A-34 and T-1190/1, were compared. Each strain was inoculated into 300 ml. of infusion broth previously heated to 37°C and having a pH of 7.15. The inocula were obtained from 24 hour blood agar plate cultures and each consisted of 3 colonies which were suspended as evenly as possible in the broth. Viable counts were performed immediately and at 3, 6, 9, 12, 15, 18, 26 and 50 hours after the commencement of incubation. The results of these counts are shown in Table 18. It will be seen that roughly comparable maximum viable counts were obtained at about the 6th to 9th hours. Thereafter the count of strain A-34 fell at a rapid rate compared with that of T-1190/1 so that by the 26th hour the difference between the two cultures in terms of living bacteria was more than 40-fold. This difference was reduced by the 50th hour. At the 26th hour the pH values of A-34 and T-1190/1 cultures were 6.45 and 6.3 respectively. Throughout the experiment both cultures showed even turbidity and the differences in opacity as judged by eye

TABLE 18

GROWTH CURVES OF STRAINS A-34 AND T-1190/1 IN BROTH

Hours after inoculation	Viable counts (millions/ml.)		Ratio of viable counts T-1190/1 / A-34
	Strain A-34	Strain T-1190/1	
0	0.085	0.155	1.8
3	4.05	4.5	1.1
6	800	950	1.2
9	1150	700	0.6
12	350	480	1.4
15	85	400	4.7
18	65	350	5.4
26	6.5	285	43.8
50	25.5	80	3.1

were negligible. Smears made at the 26th and 50th hours showed no evidence of excessive clumping.

In the light of these observations further experiments were performed to investigate comparatively the growth characteristics of 16 A strains and 15 T strains in broth cultures. The results of these experiments are summarised in Table 19. Twenty ml. volumes of broth brought to 37°C were inoculated with 1 or 2 colonies of the various strains under examination. Care was taken to suspend inocula as evenly as possible in the broths. Immediately, and at 5, 24, 48 and 72 hours after inoculation 1 ml. volumes were withdrawn from each culture for the purpose of determining viable counts and pH values. It is apparent from the Table that after inoculating broths with comparable numbers of living bacteria certain differences between A and T strains were first noticeable at the 24th hour of incubation. At this time and subsequently the pH of A cultures were slightly but consistently higher than those of T cultures. Also at 24 hours the viable counts of T strains were usually at least 10-fold greater, and certainly never less, than those of A strains. This difference in numbers of living organisms was reduced in most cases when the cultures were further incubated. At no time did cultures show evidence of clumping of bacteria. Of the A strains Nos. A-70, A-91 and A-108 gave rather higher counts in 24 hour broths than the majority. That this was not a chance result was shown by a number of repeat experiments. In 24 hour broth cultures the 3 atypical A strains repeatedly gave viable counts intermediate between those of T strains and typical A strains.

In the experiments just described no definite differences in opacity between A and T broth cultures could be seen by eye.

TABLE 19

VIABLE COUNTS AND pH VALUES OF PAST. HAEMOLYTICA BROTH CULTURES AT VARIOUS STAGES OF GROWTH.

A AND T STRAINS

Exp. No.	Strain No.	0 hours		5 hours		24 hours		48 hours		72 hours	
		Viable count (millions /ml.)	pH	Viable count (millions /ml.)	pH	Viable count (millions /ml.)	pH	Viable count (millions /ml.)	pH	Viable count (millions /ml.)	pH
1	A-34	1.3	7.15	750	6.7	13	6.6	60	6.7	90	6.7
	A-70	2.0	7.15	950	6.5	150	6.7	N	N	N	N
	A-79	2.2	7.15	750	6.65	14	6.6	N	N	N	N
	A-97	3.7	7.15	900	6.6	13	6.6	105	6.7	95	6.7
	A-114a	3.3	7.15	1400	6.6	19	6.6	24	6.6	120	6.6
	T-110	2.7	7.15	1000	6.5	350	6.3	N	N	N	N
	T-117	1.8	7.15	650	6.7	395	6.3	165	6.35	210	6.4
	T-1087/3	2.3	7.15	300	6.8	415	6.3	115	6.3	150	6.35
	T-1118	3.5	7.15	1250	6.6	390	6.3	155	6.3	150	6.3
	T-7157H	3.4	7.15	1250	6.65	295	6.3	260	6.3	390	6.4
2	A-96	2.4	7.0	850	6.6	7	6.4	12	6.6	135	6.7
	A-107	2.4	7.0	750	6.65	12	6.35	N	N	N	N
	A-108	3.5	7.0	750	6.65	115	6.4	45	6.4	160	6.4
	A-109	1.0	7.0	400	6.7	17	6.35	4	6.55	100	6.65
	A-112	2.7	7.0	1350	6.65	22	6.4	45	6.6	N	6.5
	T-86	2.2	7.0	500	6.6	600	6.2	95	6.3	150	6.35
	T-121	2.7	7.0	1000	6.6	800	6.2	165	6.2	160	6.35
	T-123	2.1	7.0	180	6.9	650	6.15	170	6.2	170	6.3
	T-124	1.8	7.0	600	6.7	900	6.2	195	6.3	150	6.3
	T-125	2.2	7.0	750	6.6	350	6.2	305	6.3	300	6.4
3	A-10	0.6	7.05	450	6.8	16	6.5	13	6.6	11	6.55
	A-36	0.7	7.05	750	6.7	2	6.4	20	6.55	4	6.6
	A-91	1.0	7.05	1300	6.55	95	6.4	45	6.45	45	6.5
	A-128	0.6	7.05	700	6.7	5	6.4	55	6.5	17	6.6
	A-2054	0.7	7.05	650	6.8	12	6.5	14	6.6	4	6.55
	A-2050	0.9	7.05	1350	6.6	20	6.35	45	6.45	80	6.5
	T-105	1.9	7.05	450	6.75	600	6.25	220	6.2	70	6.2
	T-1127	2.5	7.05	250	6.6	200	6.1	N	N	N	N
	T-1190/1	1.4	7.05	900	6.75	225	6.15	90	6.2	65	6.25
	T-2053	1.4	7.05	950	6.8	500	6.2	85	6.2	50	6.2
	T-2051	1.0	7.05	400	6.7	170	6.2	70	6.15	11	6.15

Infusion broth was used for experiments 1 and 2.

Digest broth was used for experiment 3.

N = not done.

However, to investigate this point more thoroughly an experiment was carried out on a total of 30 strains using a nephelometer. 1 to 4 colonies of various A and T strains were evenly suspended in 15 ml. volumes of infusion broth previously heated to 37°C and contained in standard glass tubes. Culture opacities were measured in a nephelometer at the 5th, 24th and 48th hours of incubation. In addition viable counts were carried out immediately after inoculation of cultures and also after 24 hours' incubation. The results are recorded in Table 20. With regard to opacities measured 5 and 24 hours after inoculation the distinction between A and T strains was complete. T strains consistently produced a slightly denser growth. It is not surprising that such a distinction could not be made by eye, particularly as the maximum opacity produced by any of the cultures was equivalent only to McFarland's Scale 0.6. By the 48th hour of incubation the opacities of a number of A and T cultures had overlapped and differentiation with certainty was no longer possible. There was no evidence of excessive clumping of bacteria in cultures.

It is apparent from the foregoing experiment that the slight differences in opacity between A and T cultures in no way accounted for the large differences in viable counts between the 2 types which occurred after 24 hours' incubation. In addition there was no evidence that clumping of A bacteria might be responsible for this effect. It would seem likely therefore that the true explanation lay in a high death rate of A organisms, compared with T organisms occurring between the 6th and 24th hours of incubation.

TABLE 20

OPACITIES AND VIABLE COUNTS OF PAST. HAEMOLYTICA BROTH CULTURES AT VARIOUS STAGES OF GROWTH.

A AND T STRAINS

		Nephelometer readings			Viable counts (millions/ml.)	
		5 hours	24 hours	48 hours	0 hours	24 hours
A Strain Nos.	A-10	33	34	42	1.9	55
	A-34	38	35	38	4.2	75
	A-36	40	40	41	3.8	50
	A-70	46	53	72	7.5	160
	A-79	38	35	36	2.2	35
	A-91	40	50	60	5.6	100
	A-96	41	40	41	2.8	50
	A-97	43	40	45	4.9	20
	A-107	40	51	60	2.5	25
	A-108	33	52	52	11.0	145
	A-109	37	40	45	1.6	18
	A-112	38	47	66	1.6	45
	A-128	43	41	48	2.2	60
	A-2054	18	32	43	1.3	35
	A-2050	38	48	68	2.9	35
Range of results for A strains		18-46	32-53	36-72	1.3-11.0	18-160
Average results for A strains		38	43	50	3.7	61
T Strain Nos.	T-86	58	64	62	2.3	600
	T-105	55	66	64	3.0	450
	T-110	54	59	54	4.6	450
	T-117	53	61	66	3.4	900
	T-121	55	68	72	1.3	750
	T-123	55	56	55	2.5	550
	T-124	54	62	61	1.4	450
	T-125	56	62	58	3.5	345
	T-1087/3	51	67	69	0.9	265
	T-1118	68	71	62	1.8	550
	T-1127	68	74	64	2.2	450
	T-1190/1	61	57	51	1.2	185
	T-7157H	52	66	63	1.4	450
	T-2053	59	67	68	1.9	650
	T-2051	54	59	62	4.9	800
Range of results for T strains		53-68	56-74	51-72	0.9-4.9	185-900
Average results for T strains		57	64	62	2.4	523

Nephelometer set so that standard suspension (McFarland's Scale 0.5) gave a reading of 60.

Nephelometer readings are in direct proportion to culture opacities.

(d) Sensitivity of A and T strains to Antibiotics

A number of A and T strains were tested using Multodisks as already described. The results are recorded in Table 21. Clear cut differences between A and T strains became apparent using penicillin and tetracycline. A strains were the more sensitive to each of these two antibiotics. Penicillin gave a particularly obvious differentiation since under the conditions of the experiment this substance failed completely to inhibit the growth of T strains.

Examination for sensitivity to penicillin by the broth dilution method gave the results shown in Table 22. In the presence of 0.1562 units of penicillin per ml. of broth all of 15 T strains produced visible growth after 18 hours incubation while only one of 15 A strains, No. A-10, did so. It may be worthy of note that this strain was obtained from the nasal passages of a healthy sheep and not from a case of disease. It gave the same result when retested. All strains examined grew in the presence of 0.0781 units of penicillin per ml. A level of 0.625 units per ml. was necessary to ensure the suppression of visible growth of T strains incubated for 18 hours. Some but not all of the tubes in which growth was inapparent proved to be bacteriologically sterile.

(e) Source of Cultures

A considerable number of strains isolated from diseased sheep have now been classified as A or T types. There appears to be a high degree of correlation between particular forms of disease and the type of Past. haemolytica isolated.

Fifty carcases of sheep and lambs obtained from a variety of

TABLE 21

ANTIBIOTIC SENSITIVITY OF A AND T STRAINS

		Zones of inhibition in millimetres produced by antibiotics					
		C	E	OL	S	TE	P
A Strain Nos.	A-34	6.5	2.5	0	0.5	4	2
	A-70	5.5	3	0	1	4.5	2.5
	A-79	7	3	0	0	4.5	3
	A-82B	6.5	2.5	0	1	5	4.5
	A-91	6	2.5	0	0.5	4.5	2.5
	A-92	6.5	3	0	0.5	4.5	1
	A-95	6.5	1.5	0	0.5	4.5	2.5
	A-97	7	3.5	0	0.5	4.5	3.5
	A-99	6.5	4	0	0.5	4	2.5
	A-103	5	2.5	0	0.5	4	2
	A-108	7.5	4	0	0.5	5	2
T Strain Nos.	T-89B	5	1.5	0	0.5	3	0
	T-94	4.5	1	0	0	3.5	0
	T-100C	5.5	2	0	0	3.5	0
	T-105	5	1.5	0	0	2.5	0
	T-1087/3	5	1.5	0	0	2.5	0
	T-1118	4	1	0	0	2.5	0
	T-1127	5.5	2	0	0	3.5	0
	T-1190/1	5	1.5	0	0.5	3.5	0
	T-7157H	5	1.5	0	0	3.5	0
	T-2053	4.5	2	0	0.5	3	0
	T-2056	6.5	2	0	0	2.5	0
	T-2058	6	2.5	0	0	3.5	0

Each measurement represents the distance in millimetres between the edge of the paper disc and the circumference of the inhibition zone.

C = Chloromycetin
E = Erythromycin
OL = Oleandomycin
S = Streptomycin
TE = Tetracycline
P = Penicillin

TABLE 22

PENICILLIN SENSITIVITY OF A AND T STRAINS

		Effect of different concentrations of penicillin on growth in broth									
		1.25u/ml.		0.625u/ml.		0.3125u/ml.		0.1562u/ml.		0.0781u/ml.	
		V.G.	L.O.	V.G.	L.O.	V.G.	L.O.	V.G.	L.O.	V.G.	L.O.
A Strain Nos.	A-10	-	-	-	-	-	-	+	+	+	+
	A-34	-	-	-	-	-	-	-	+	+	+
	A-36	-	-	-	-	-	-	-	+	+	+
	A-70	-	-	-	-	-	-	-	-	+	+
	A-79	-	-	-	-	-	-	-	+	+	+
	A-91	-	-	-	-	-	-	-	-	+	+
	A-96	-	-	-	-	-	-	-	+	+	+
	A-97	-	-	-	-	-	-	-	+	+	+
	A-107	-	-	-	-	-	-	-	-	+	+
	A-108	-	-	-	-	-	-	-	-	+	+
	A-109	-	-	-	-	-	-	-	-	+	+
	A-112	-	-	-	-	-	-	-	+	+	+
	A-128	-	-	-	-	-	-	-	-	+	+
	A-2054	-	-	-	-	-	-	-	+	+	+
	A-2050	-	-	-	-	-	-	-	-	+	+
T Strain Nos.	T-86	-	-	-	-	-	-	+	+	+	+
	T-105	-	-	-	-	+	+	+	+	+	+
	T-110	-	-	-	-	+	+	+	+	+	+
	T-117	-	-	-	-	-	-	+	+	+	+
	T-121	-	-	-	-	+	+	+	+	+	+
	T-123	-	-	-	-	-	+	+	+	+	+
	T-124	-	-	-	-	-	-	+	+	+	+
	T-125	-	-	-	-	+	+	+	+	+	+
	T-1087/3	-	-	-	-	-	+	+	+	+	+
	T-1118	-	-	-	+	+	+	+	+	+	+
	T-1127	-	-	-	+	-	+	+	+	+	+
	T-1190/1	-	-	-	-	-	-	+	+	+	+
	T-7157H	-	-	-	-	+	+	+	+	+	+
	T-2053	-	-	-	-	-	-	+	+	+	+
	T-2051	-	-	-	-	-	-	+	+	+	+

Tests read after 18 hours incubation.

Columns marked V.G. record presence or absence of visible growth.

Columns marked L.O. record presence or absence of living organisms.

sources and showing lesions of enzootic pneumonia as described by Montgomerie, Bosworth and Glover (1958) have been examined. The infection was usually confined to the lung but occasionally a bacteraemia was present and very much smaller numbers of organisms could be cultured from one or more of several other sites, namely, heart blood, liver, spleen and kidney. Type A organisms in pure culture were obtained from 40 cases. One case yielded a few A organisms and a luxuriant growth of C. pyogenes. One case yielded a T strain together with C. pyogenes, the latter predominating. From the remaining 8 cases mixtures of A and T strains were isolated and in one of these C. pyogenes was present also.

Strains from 15 different outbreaks of septicaemia as described by Stamp, Watt and Thomlinson (1955) were examined. The animals involved were 6 to 12 months old. Each strain proved to be of the T variety and no A strains have as yet been isolated from septicaemias in lambs of this age group.

In the spring of 1959 eighteen cases of a septicaemic type of disease in very young lambs were investigated. Most of these occurred in the Moredun Institute flock. The majority of lambs involved were 3 to 5 weeks of age and none was older than 12 weeks. Seventeen yielded A strains and 1 yielded a T strain.

B. INTRAVENOUS INOCULATION OF SHEEP WITH LIVING CULTURES OF PASTEURELLA HAEMOLYTICA TYPES A AND T

The primary object of this experiment was to investigate the possibility of differences in behaviour between A and T strains following intravenous injection into sheep. Two possibilities were visualised, namely (1) differences in lethal capacities of the two types when inoculated in roughly comparable doses, and (2) differences in the abilities of A and T strains to multiply *in vivo* as judged by the numbers of viable bacteria present in various tissues at death. Bearing in mind the high cost of the sheep as an experimental animal any such difference would have to be clear cut to enable a reasonably large sample of A and T strains to be tested.

MATERIALS AND METHODS

Strains. 6 strains derived from cases of ovine pneumonia and septicaemia and previously typed as A or T strains by the methods described earlier were used as shown in Table 23. For further details of the strains reference may be made to Appendix I.

Sheep. 7 sheep whose ages ranged from 7 months to 2 years were used as shown in Table 23. The experiments were performed on different occasions within a period of 6 weeks.

Inocula. These are described in Table 23. They consisted either of 5½ hour infusion broth cultures or of suspensions of 17 hour growths on blood agar in 1% casein hydrolysate solution. The latter were stored at +4°C for 24 hours before use. Viable counts of inocula were made immediately before injection.

Blood cultures. Wherever these were performed the method used was to

inoculate two 10 ml. volumes of broth with 5 ml. and 1 ml. respectively of blood. After 24 hours incubation these cultures were examined by appropriate means for the presence of Past. haemolytica.

Bacterial Counts on Tissues. These were performed on the lung, liver, spleen and kidney of each sheep which died. The methods used were those previously described on Page 53.

RESULTS

The sheep were examined clinically at various intervals after inoculation and the observations made are noted below.

Sheep 1

3½ hours: The temperature had risen from 103.4°F to 106.4°F. No other symptoms were present.

18 hours: Temperature 103°. The sheep appeared healthy except for a slight lameness in the right foreleg.

No further symptoms were shown but the lameness persisted for several days.

Sheep 2

7 hours: The temperature had risen from 103.7° to 104.2°. Respirations were accelerated and a severe lameness in the right hind leg was observed. The animal was bright.

18 hours: Temperature 104.4°. The sheep was prostrate and unable to rise. Respirations were accelerated and auscultation of the chest indicated the presence of

pleural exudate. Blood culture gave rise to Pasteurella.

19½ hours: Died.

A post-mortem examination was performed. The trachea and bronchi were filled with frothy fluid. The pleural cavity was approximately quarter full of clear fluid. Sub-pleural haemorrhages averaging 5 mm. in diameter covered the surfaces of both lungs. Two small patches of consolidation about 4 cm. in diameter were present, one on the right apical lobe, the other on the inferior border of the right diaphragmatic lobe. The cut surface of the lung revealed that small haemorrhagic areas, frequently associated with the bronchi, were present in the lung substance. A considerable amount of frothy exudate could be expressed from the cut surface. The epicardium showed a small number of petechiae. The abomasal mucosa was completely covered with petechial haemorrhages. The intestinal blood vessels were slightly injected. Histologically the lungs showed early acute bronchopneumonia with congestion, alveolar oedema and peri-bronchial lymphoid hyperplasia. No organisms were visible. Liver sections indicated mild fatty change and areas of the white pulp of the spleen showed degenerative changes including karyorrhexis. The results of viable counts of bacteria in various tissues at death are shown in Table 24.

Sheep 3

4½ hours: The temperature had risen from 103.3° to 107.2°. The sheep appeared normal.

9½ hours: Temperature 104.2°. No other symptoms were observed.

18 hours: Temperature 105.2°. The animal was eating and appeared bright. A blood culture proved to be negative.

34 hours: Temperature 103.9°.

No further symptoms were observed.

Sheep 4

5½ hours: The temperature had risen from 102.8° to 106.6° but no other abnormal signs were present.

18 hours: Temperature 105°. A slight lameness had developed in the right foreleg but in other respects the animal appeared normal. However a blood culture was positive for Past. haemolytica.

26 hours: Temperature 105.4°. Again there were no obvious signs of illness and the animal was ruminating but Pasteurella were cultured from the blood.

48 hours: Temperature 103°. The lameness had disappeared and the sheep appeared well.

No further symptoms were shown.

Sheep 5

2½ hours: The temperature had risen from 104.1° to 105.4° but no other signs of illness were present.

7 hours: Temperature 106.8°. The respiration rate was 136 per minute. The sheep was listless and obviously ill.

24 hours: Temperature 105.8°. Acute lameness in the right foreleg and listlessness were observed. A blood culture yielded Pasteurella.

48 hours: Temperature 105.7°. The animal was eating but showed acute lameness in several legs. A blood culture was negative.

72 hours: Temperature 105.2°. Severe lameness was still present but there were no other symptoms. The animal was killed.

At post-mortem examination no obvious abnormalities were detected anywhere in the carcase, including the joints. Cultures made from lung, liver, spleen, kidney, heart blood and joints were all negative.

Sheep 6

7 hours: The temperature had risen from 102.8° to 105.4°. The animal was very ill and refused to stand. Severe diarrhoea was present.

15 hours: Died.

Post-mortem examination showed haemorrhages on the inner and outer surfaces of the trachea. Ecchymoses were present on the endocardium. The abomasal mucosa was covered with haemorrhages and the intestines were inflamed. Viable counts of bacteria in tissues are given in Table 24.

Sheep 7

7 hours: The temperature had risen from 102.6° to 107.3°. The animal was bright but diarrhoea was present.

16½ hours: Died.

At post-mortem examination the abnormalities detected were as for sheep No. 6 but in addition the lungs were a slate blue colour. Table 24 shows the numbers of viable organisms present in tissues.

TABLE 23

INTRAVENOUS INOCULATION OF SHEEP WITH STRAINS OF PAST. HAEMOLYTICA.

TECHNICAL DATA AND RESULTS

Sheep No.	Age of sheep	Strain of Past. haemolytica	Type of strain	Inoculum	Result
1	7 months	2054	A	5 ml. broth culture 2035×10^6 viable organisms	Survived
2	7 months	34	A	10 ml. broth culture 4650×10^6 viable organisms	Died
3	7 months	2053	T	5 ml. blood agar culture suspension 970×10^6 viable organisms	Survived
4	7 months	2053	T	5 ml. blood agar culture suspension 3550×10^6 viable organisms	Survived
5	19 months	105	T	5 ml. blood agar culture suspension 1955×10^6 viable organisms	Survived
6	19 months	70	A	5 ml. blood agar culture suspension 14250×10^6 viable organisms	Died
7	19 months	1190/1	T	5 ml. blood agar culture suspension 18000×10^6 viable organisms	Died

TABLE 24

VIABLE COUNTS OF PAST. HAEMOLYTICA IN TISSUES OF SHEEP WHICH DIED AFTER INTRAVENOUS INOCULATION

Sheep No.	Viable counts in various organs			
	Lung	Liver	Spleen	Kidney
2	18.0	0.06	10.7	3.2
6	0.13	0	0	0
7	3.7	0.5	13.5	12.5

Viable counts given in millions per gm. of tissue

Certain technical data and also the results of these experiments are summarised in Tables 23 and 24. While 4 of the 7 sheep inoculated survived, the 3 which succumbed yielded numbers of bacteria from lung, liver, spleen and kidney tissue which indicated that no marked bacterial multiplication had occurred. In one case liver, spleen and kidney were bacteriologically sterile. There was no evidence of differences in behaviour between A and T strains following intravenous inoculation.

PART 5

PATHOGENICITY OF PAST. HAEMOLYTICA FOR YOUNG LAMBS

In the spring of 1959 a number of deaths in very young lambs was investigated and found to be associated with generalised infection of Past. haemolytica. The lambs involved varied from 2 days to 12 weeks in age but the majority were 3 to 4 weeks old. Twelve cases occurred in the Moredun Institute flock and in addition 6 cases from other sources were examined. The majority showed fibrinous pleurisy with adhesions to the chest wall and small areas of pneumonia were usually associated with these lesions. Pericarditis was sometimes present and several lambs showed subepicardial, pericardial and sub-pleural haemorrhages, the latter occurring beneath both visceral and parietal pleura. One lamb showed acute fibrinous peritonitis in addition to thoracic lesions. In 2 cases acute fibrinous peritonitis with adhesions and turbid exudate was the only visible abnormality. The liver of one lamb showed numerous subcapsular, pin-point, whitish foci.

On surface culture pneumonic lung tissue invariably yielded heavy confluent growth of Past. haemolytica. Slightly less luxuriant but still very considerable growth was obtained from apparently normal lung tissue, liver and spleen. Kidney cultures were always positive but the numbers of bacteria present were often much smaller. The organism could be regularly isolated from the tracheal mucous membrane.

Of the 18 cases examined 17 yielded Past. haemolytica Type A and only 1 yielded Type T. This is in contrast to the septicaemic disease of older lambs in which, as has already been stated, Type T

organisms appear to be usually if not invariably involved. Work described previously showed that Type A infections in older animals are virtually confined to the thoracic cavity. The recognition of generalised Type A infections in young lambs suggested that these animals might be particularly susceptible to experimental infections with a freshly isolated A strain. Investigation of this point constituted the main purpose of the following series of experiments.

MATERIALS AND METHODS

Strains. Two strains of Past. haemolytica were used.

Strain 158 was a Type A organism obtained from a 3 week old lamb which died at the Moredun Institute during the 1959 outbreak. At post-mortem the only macroscopic abnormalities in this lamb consisted of a few sub-pleural haemorrhages on the chest wall and numerous subcapsular, pin-point, whitish foci in the liver. Histologically the liver showed numerous small foci of organisms scattered throughout the parenchyma, apparently in the sinusoids, and in every case surrounded by an area of acute, cellular necrosis. No further cellular reaction was present. Similarly the lung showed many foci of bacteria in the alveolar walls, probably within capillaries. Cellular reaction in the vicinity of these clumps was very slight. Counts of viable bacteria present at death in lung and liver tissue gave the results 207×10^6 and 750×10^6 per gram respectively.

Strain 7157H was a Type T organism maintained for several years in the laboratory. It was preserved in the freeze-dried state ready for use after a single mouse passage. Further details are given in Appendix I.

Experimental Animals. Three to 4 week old lambs were obtained from two farms for use in these experiments. The adult sheep used consisted of 1 and 2 year old animals from the Institute flock. Mice were approximately 4 weeks old at the time of inoculation.

Intraperitoneal and Intravenous Inocula. These consisted of dilutions of 5 hour infusion broth cultures. Immediately before injection viable counts of bacteria in inocula were performed by the method described in Appendix II. The average of 3 replicate counts was taken to be the true value.

Intratracheal Inoculations. Inocula consisted either of 5 hour infusion broth culture or of suspensions of 18 hour blood agar culture in aqueous 1% casein hydrolysate. Injections were carried out under deep ether anaesthesia.

Blood Cultures. 1 ml. volumes of blood were withdrawn aseptically from the jugular vein and inoculated into 10 ml. volumes of broth. After incubation these cultures were examined for the presence of Past. haemolytica.

Counts of Viable Bacteria in Tissues. Counts were performed on various organs and body fluids at death. The method used has already been described the only difference being that in the case of spleen, by virtue of the small size of the organ, it was necessary to prepare the tissue suspension from 1 gm. of tissue instead of 5 gm. No replicate counts were carried out.

RESULTS

(a) Preliminary Pathogenicity Test using Strain 158

Two lambs were inoculated with the freshly isolated Strain 158, one intraperitoneally and the other intravenously. A primary culture on blood agar was used to inoculate infusion broth which was then incubated for 5 hours. The dose of broth culture per lamb was 5 ml. of a 1/50 dilution and this was shown to contain 11.5×10^6 viable bacteria.

Lamb 1. This animal was inoculated intraperitoneally and it died after 12 hours from an acute, fibrinous peritonitis. The abdominal cavity contained about 30 ml. of pale amber, turbid fluid. Strands of fibrin were present. The omentum was congested and slightly adherent to the abdominal wall. The diaphragmatic surface of the liver was encased in fibrin. The peritoneal coat of the caecum and large intestine showed early inflammatory changes. The small intestines were congested and the reddened Peyer's patches were enlarged and visible from the exterior. In the caecum and large intestine the mucosa was thrown into folds and ridges, the summits of which were markedly congested. The thorax contained approximately 20 ml. of slightly turbid, amber fluid and a small number of sub-pleural haemorrhages was present on the chest wall. The lungs were apparently normal except for a few small, sub-pleural haemorrhagic spots and several tiny areas of collapse. Counts of viable bacteria in tissues and fluids gave the results shown below.

Lung	0.5×10^6 per gm.
Liver	0.1×10^6 " "
Spleen	0.2×10^6 " "
Kidney	3.0×10^6 " "
Peritoneal exudate	18000.0×10^6 per ml.
Pleural exudate	205.0×10^6 " "

Lamb 2. The inoculum was given intravenously into the left jugular vein. Next day the lamb was acutely lame on the right foreleg. Its temperature was 106.9°F and a blood culture demonstrated the presence of circulating Past. haemolytica. The following day the right foreleg was extremely swollen and painful and the animal showed a temperature of 106.2°. It was slaughtered on humane grounds. On post-mortem the only abnormality seen was in the affected foreleg in which a severe purulent arthritis was present in the knee and fetlock. The copious greenish-white pus yielded a pure culture of Past. haemolytica. Lung, liver and kidney were bacteriologically sterile and a plug of spleen tissue taken with a Pasteur pipette yielded only one colony on culture.

(b) Intraperitoneal Inoculation of Young Lambs and Adult Sheep with Graded Doses of Strain 158

A primary blood agar culture of Strain 158 derived from Lamb 1 was used to inoculate infusion broth which after 5 hours incubation contained 290×10^6 viable organisms per ml. The various dose levels used were prepared from this broth culture. Pairs of lambs were inoculated with doses of 58×10^6, 58×10^4, 58×10^2 and 58 viable bacteria contained in dose volumes of 2.0 ml. Pairs of adults were inoculated in the same way except that the smallest dose was omitted. Each pair of adults consisted of a 1 year old and a 2 year old sheep.

The results are shown in Table 25. All deaths occurred within 24 hours of inoculation. The survivors were kept for 7 days before being discarded. It is clear that the organism was of high virulence for the lambs amongst which 6 deaths occurred. Prior to death

TABLE 25

INTRAPERITONEAL VIRULENCE TITRATIONS OF STRAIN 158 IN YOUNG LAMBS AND ADULT SHEEP

Dose of viable organisms	Deaths	
	Lambs	Adults
58×10^6	$^2/_2$	$^0/_2$
58×10^4	$^1/_2$	$^0/_2$
58×10^2	$^2/_2$	$^0/_2$
58	$^1/_2$	N

N = Not done.

TABLE 26

BACTERIOLOGICAL EXAMINATION OF 6 LAMBS WHICH DIED AFTER INTRAPERITONEAL INJECTIONS OF STRAIN 158

Doses of viable bacteria received by 6 lambs (millions)	Viable counts of bacteria in tissues and exudates (millions per gm. or ml.)			
	Lung	Liver	Peritoneal exudate	Pleural exudate
58.0	1.0	0.007	18,500.0	26.0
58.0	375.0	5.0	45,000.0	17,000.0
0.58	4.0	0.1	27,500.0	3,500.0
0.0058	12.0	4.0	45,000.0	150.0
0.0058	N	N	15,500.0	N
0.000058	N	N	2,350.0	N

N = Not done.

elevated temperatures ranging from 105.3° to 108°F. were observed. The adult sheep were completely resistant to the doses used and no elevated temperatures, symptoms or deaths occurred in these animals. The post-mortem appearances of the dead lambs were essentially similar to that of Lamb 1. Acute, fibrinous peritonitis was present and the quantities of peritoneal exudate varied from approximately 10 ml. to 100 ml. The volumes of pleural exudate ranged from 5 ml. to 20 ml. Counts of viable bacteria in various tissues and exudates were carried out and are recorded in Table 26. Peritoneal fluids contained enormous numbers of organisms and it is clear that multiplication in vivo occurred to a very high degree.

(c) Intraperitoneal Inoculation of Young Lambs and Adult Sheep with Graded Doses of Strain 7157H

As mentioned earlier Strain 7157H had been maintained for a considerable period in the laboratory. The experimental design and methods were precisely similar to those used in the previous experiment. In this case however the 4 dose levels contained 140×10^6, 140×10^4, 140×10^2 and 140 viable organisms. Only one death was produced and this in a lamb which received the largest dose. It died with acute, fibrinous peritonitis 12 hours after inoculation. One lamb which had received 140×10^4 organisms showed a temperature of 105.3° next day but this rapidly subsided. None of the other lambs or adults showed a temperature rise or symptoms. After 7 days survivors were discarded. The results of viable counts of organisms in tissues and exudates of the dead lamb were as follows:-

Lung	57×10^6 per gm.
Liver	0.07×10^6 " "
Spleen	0.2×10^6 " "
Peritoneal exudate (15 ml. present)	$8,500 \times 10^6$ per ml.
Pleural exudate (20 ml. present)	900×10^6 " "

(d) Intravenous Inoculation of Young Lambs, Adult Sheep and Mice with Graded Doses of Strain 158

Pooled peritoneal exudates from the lambs whose deaths are recorded in Table 25 were cultured on blood agar. A single colony was then inoculated into infusion broth which after 5 hours incubation contained 670×10^6 viable organisms per ml. This broth culture was used to prepare various doses for animal inoculation. Four dose levels were used and these consisted of 335×10^6, 335×10^4, 335×10^2 and 335 living bacteria. The smallest dose was tested intravenously in a group of 3 lambs. The 3 larger doses were each tested intravenously in groups of 3 lambs, 2 adult sheep and 6 mice, and intraperitoneally in a group of 6 mice. The dose volumes used were 5.0 ml. for lambs and sheep and 0.5 ml. for mice.

Table 27 shows the fates of the inoculated animals. Seven lambs died but all adult sheep and mice were resistant to the doses used. On the day after inoculation the adults showed normal temperatures and blood cultures were negative. Mice which received the largest dose level intraperitoneally lost weight and showed signs of illness in the following 48 hours but they rapidly recovered. In all other mice the inocula produced no observable effect. Three of the 7 lambs which died were animals which had received the largest dose level and they succumbed within 24 hours of inoculation. The

TABLE 27

VIRULENCE TITRATIONS OF STRAIN 158 IN LAMBS, ADULT SHEEP AND MICE

Dose of viable bacteria	Deaths and survivals			
	Inoculated intravenously			Inoculated intraperitoneally
	Lambs	Adult sheep	Mice	Mice
335×10^6	Lamb No. 11 D " " 12 D " " 13 D	$^2/_2$ S	$^6/_6$ S	$^6/_6$ S
335×10^4	Lamb No. 14 D " " 15 D " " 16 D	$^2/_2$ S	$^6/_6$ S	$^6/_6$ S
335×10^2	Lamb No. 17 S " " 18 S " " 19 S	$^2/_2$ S	$^6/_6$ S	$^6/_6$ S
335	Lamb No. 20 D " " 21 S " " 22 S	N	N	N

D = Died.

S = Survived.

N = Not done.

Lambs No. 11, 12 and 13 died within 24 hours of inoculation.

Lambs No. 14, 15, 16 and 20 died 8, 16, 7 and 13 days respectively after inoculation.

Lambs No. 14, 15 and 20 yielded Actinobacillus lignieresi in addition to Past. haemolytica on culture.

TABLE 28

BLOOD CULTURES IN LAMBS INOCULATED INTRAVENOUSLY WITH STRAIN 158

Lamb Nos.	Blood cultures at intervals after inoculation							
	2 days	3 days	6 days	7 days	9 days	10 days	13 days	20 days
11	D	D	D	D	D	D	D	D
12	D	D	D	D	D	D	D	D
13	D	D	D	D	D	D	D	D
14	+	+	-	-	D	D	D	D
15	+	-	-	-	-	-	-	D
16	+	+	+	D	D	D	D	D
17	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-
19	-	-	-	+	+	-	+	-
20	-	-	-	-	-	-	+	D
21	+	-	-	-	-	-	-	-
22	+	-	-	-	-	-	-	-

+ = Past. haemolytica present.

- = " " absent.

D = Lamb dead.

Each culture consisted of 1.0 ml. blood in 10.0 ml. broth.

Inocula are described in Table 27.

TABLE 29

BACTERIOLOGICAL EXAMINATION OF LAMBS WHICH DIED OR WERE SLAUGHTERED AFTER INTRAVENOUS INJECTIONS OF STRAIN 158

Lamb Nos.	Viable counts of Past. haemolytica in tissues and exudates (millions per gm. or ml.)					
	Lung	Liver	Spleen	Kidney	Peritoneal exudate	Pleural exudate
11	0.9	0.05	6.2	0.2	12,000.0	285.0
12	102.0	10.0	65.0	2.5	20,000.0	N
13	0.07	0.4	12.5	0.2	6,500.0	7,500.0
14	F ¤	F ¤	0.07	F ¤	21,500.0	N
15	550.0	1.2¤	67.0	N	0 ¤	0 ¤
16	1100.0	1075.0	6.0	0	0.03	1.2
17	0	0	0	0	0	0
18	0	0	0	0	0	0
19	1.1	0	0	0	0.7	N
20	250.0	10.2	4.2	N	0 ¤	N
21	0	0	0	0	0	0
22	0	0	0	0	0	0

Inocula and fate of lambs described in Table 27.

Where pulmonary lesions present viable counts were performed on abnormal lung tissue.

F = Past. haemolytica present, but less than 0.02×10^6 per gm. of tissue.

¤ = Actinobacillus lignieresi present.

N = Not done.

four other deaths occurred in lambs receiving smaller doses and were delayed for periods ranging from 8 to 16 days after inoculation. The surviving lambs were slaughtered and examined after 22 days at which time they showed no outward signs of illness. Blood cultures were performed at intervals throughout the experiment and the results which are recorded in Table 28 show that circulating bacteria could be demonstrated at times in the majority of lambs. Many of the lambs destined to die showed fluctuating temperatures which were frequently normal. Counts of viable bacteria in the tissues and exudates of lambs which died and of those which were slaughtered are shown in Table 29. It will be seen that 3 of the lambs which died yielded Actinobacillus lignieresi in addition to Past. haemolytica on culture. Post-mortem observations and in some cases clinical data concerning individual lambs are given below. The lambs are identified as in Table 27 from which information regarding size of infecting dose may be obtained.

Lambs 11, 12 and 13. Death from acute, fibrinous peritonitis occurred within 24 hours of inoculation. The post-mortem appearance of each lamb was essentially similar to that of Lamb 1 which has already been described. Volumes of peritoneal exudate varied from approximately 30 ml. to 60 ml. Two of the three lambs showed petechiation of the abomasal mucosa. Enormous numbers of bacteria were present in peritoneal exudates.

Lamb 14. Death took place 8 days after inoculation. Pleurisy with adhesions but only a trace of exudate was present. The apical lobe of the right lung showed areas of consolidation and was covered with

a layer of fibrin. Both lungs were generally congested and purplish in colour with a number of sub-pleural haemorrhages. The pericardium was thickened and covered with petechiae. Haemorrhages were also present under the epicardium, on the thoracic surface of the diaphragm and under the parietal peritoneum. There was marked peritonitis with fibrin, adhesions and about 10 ml. of cloudy exudate. This exudate contained Past. haemolytica in profuse numbers. Actinobacillus lignieresi was also isolated from the carcase.

Lamb 15. On the day following inoculation this animal showed lameness which became progressively worse until after 14 days both hind legs were virtually paralysed. Severe respiratory symptoms appeared shortly before death which occurred 16 days after inoculation. On post-mortem marked fibrinous pleurisy with adhesions was seen. A considerable amount of greenish cloudy pleural exudate was present. The lungs were slate-blue in colour and showed sub-pleural haemorrhages but there were no areas of consolidation. The pericardial sac contained a small amount of fibrinous exudate. There was a fibrinous peritonitis with adhesions and several hundred millilitres of cloudy, greenish fluid. A spinal abscess several centimetres in diameter was found in the lumbar region and this yielded a mixture of Past. haemolytica and Actinobacillus lignieresi on culture. The latter organism was isolated from several other sites. Lung and spleen tissue yielded large numbers of Past. haemolytica.

Lamb 16. Slight lameness was shown on the day following inoculation and persisted until death which occurred after 7 days. At post-mortem

subcutaneous blood vessels were seen to be injected. Fibrinous pleurisy with adhesions and about 15 ml. of blood-tinged pleural exudate was present. The right lung was covered with fibrin which was easily stripped off to show surface haemorrhages and generalised congestion of lung tissue. Consolidation and collapse were present in the apical lobe only. The left lung was pink and normal in appearance. Fibrinous pericarditis with a considerable amount of exudate and sub-epicardial haemorrhages were observed. There was a fibrinous peritonitis with adhesions and about 60 ml. of clearish fluid. Very large numbers of pin-point, whitish foci were present on the surface and in the substance of the liver. These lesions were similar to those present in the original lamb from which the experimental strain of Past. haemolytica was isolated. The surface of the liver showed many haemorrhages. The intestines were inflamed. No lesions were visible in joints. An abscess found in the cortex of one kidney yielded Past. haemolytica. Very large numbers of this organism were present in tissue removed from both consolidated and non-consolidated areas of the right lung and from the liver.

Lamb 17. This animal was slaughtered 22 days after inoculation. The only abnormality observed was the presence of about 10 ml. of clearish peritoneal fluid. The carcase was bacteriologically sterile.

Lamb 18. When slaughtered 22 days after inoculation the only visible abnormality consisted of a number of whitish foci, 0.25 cm. in diameter, on the surface and in the substance of the liver. These and other parts of the carcase were bacteriologically sterile.

Lamb 19. This lamb was also slaughtered 22 days after inoculation. The left lung showed fibrinous pleurisy with adhesions and an underlying pneumonia affecting the apical and cardiac lobes and part of the diaphragmatic lobe. In the abdominal cavity a few strands of fibrin were present together with about 10 ml. of cloudy fluid. A considerable number of haemorrhages under the parietal peritoneum were observed. Pneumonic lung tissue and peritoneal exudate contained relatively small numbers of Past. haemolytica. Other sites cultured were bacteriologically sterile.

Lamb 20. Death occurred 13 days after inoculation. Symptoms of lameness had been observed. A thick, extensive layer of fibrin was present on the left lung and there were adhesions to the chest wall. After removal of the fibrin the lung was seen to be congested and covered with numerous small haemorrhagic areas. Many tiny, whitish foci were scattered throughout the tissue of both lungs. There was a severe peritonitis, the intestinal folds being firmly stuck together with rubbery material. Approximately 5 to 10 ml. of cloudy fluid were present in the abdominal cavity. The liver was completely encased in fibrin and a number of whitish lesions up to 0.5 cm. in diameter were seen on the surface and in the substance of the organ. The abomasal mucosa showed several large haemorrhagic areas. The joints were apparently normal. Past. haemolytica was isolated from several sites and large numbers were present in lung tissue. Actinobacillus lignieresi was cultured from peritoneal exudate.

Lamb 21. The animal was slaughtered 22 days after inoculation.

On post-mortem the only abnormalities consisted of about 5 ml. of cloudy peritoneal fluid and several lesions on the surface of the liver which took the form of small abscesses up to 1 cm. in diameter. These were frequently surrounded by a zone of tiny, haemorrhagic spots. The carcase was bacteriologically sterile.

Lamb 22. As for Lamb 17.

Morphological and cultural characteristics together with reactions in the presence of 24 fermentable substances were determined for cultures of Past. haemolytica isolated from the carcases of Lambs 14, 19 and 20. The properties were in each case found to be those of the strain inoculated.

The results just described may be summed up as follows. The largest dose level of Strain 158 produced rapidly fatal peritonitis following intravenous inoculation. Smaller doses frequently resulted in protracted but eventually fatal infections and on post-mortem lesions similar to those frequently encountered in naturally occurring cases were present in various combinations. Several of the lambs which succumbed after prolonged infections yielded Actinobacillus lignieresi as well as Past. haemolytica on culture.

(e) Intratracheal Inoculation of Young Lambs with Strain 158

Lambs No. 23, 24 and 25 were inoculated intratracheally. Lamb 23 received 1 ml. of the 5 hour broth culture used in the previous experiment and this dose contained 670×10^6 viable organisms. The same broth culture was used as inoculum for a blood agar culture from which, after 18 hours incubation, a suspension of Past. haemolytica in 1% casein hydrolysate solution was prepared. This suspension

contained 13,000 x 10^6 viable bacteria per ml. and was administered to Lambs 24 and 25 in doses of 1 ml. and 2 ml. respectively.

Lamb 23. Following inoculation this animal showed no symptoms of ill-health and blood cultures were consistently negative. After 22 days the lamb was slaughtered and on post-mortem examination the apical and cardiac lobes of the right lung were found to be pneumonic. The lesion which in extent was similar to that frequently seen in enzootic pneumonia was pale red in colour and appeared to be resolving. Pneumonic lung tissue contained the relatively small number of 0.85 x 10^6 viable organisms per gram. Liver, spleen and kidney were bacteriologically sterile.

Lamb 24. A temperature of 106.2°F was observed and a blood culture was positive on the day after inoculation. After a further 24 hours the temperature had become normal. Several of the blood cultures performed subsequently proved to be positive and the lamb died 7 days after inoculation. On post-mortem lesions of enzootic pneumonia were seen. The apical and cardiac lobes of the right lung were deep red, consolidated and covered with a thick layer of fibrin. The remainder of the right lung was slightly congested but the left lung appeared normal. Adhesions were present in the chest but there was little pleural exudate. A fibrinous pericarditis was observed. In the abdominal cavity there was a fibrinous peritonitis with adhesions and about 25 ml. of cloudy exudate. Counts of Past. haemolytica in various tissues and exudates gave the results shown below.

Apical lobe of right lung	80 x 10^6 per gm.
Diaphragmatic lobe of left lung	165 x 10^6 " "

Liver	55×10^6 per gm.
Spleen	375×10^6 " "
Kidney	0.07×10^6 " "
Peritoneal exudate	$5,500 \times 10^6$ per ml.

Lamb 25. Respiratory symptoms were apparent 24 hours after inoculation and the lamb showed a temperature of 106.9°. Death took place on the second day and prior to this blood culture had demonstrated the presence of circulating organisms. Post-mortem examination showed severe pleurisy with many adhesions and several hundred millilitres of exudate. The left lung was entirely covered with fibrin. Both lungs showed a generalised, livid red colour and interlobular oedema. Small dark red areas of consolidation were scattered over lung surfaces and both diaphragmatic lobes exhibited circumscribed lesions of pneumonia. The mediastinal tissues were swollen with gelatinous oedema and there was a fibrinous pericarditis with a considerable amount of exudate. Pericardial and sub-epicardial petechiation was observed. More than 100 ml. of clearish fluid were present in the abdominal cavity but there was no fibrin. Tissue surrounding the spleen and kidneys was swollen with gelatinous oedema. A few tiny haemorrhages were scattered over the abomasal mucosa but these may have been due to worms which were present in considerable numbers. Viable counts of Past. haemolytica in various tissues and exudates gave the following results.

Lung	4×10^6 per gm.
Liver	1×10^6 " "
Spleen	12×10^6 " "
Kidney	0.1×10^6 " "
Pleural exudate	$15,000 \times 10^6$ per ml.

Pericardial exudate	1×10^6 per ml.
Peritoneal exudate	0.6×10^6 " "

Using the methods of examination outlined in the preceding section isolates from Lambs 23 and 24 appeared to be identical to the strain of Past. haemolytica inoculated.

PART 6

IMMUNOLOGICAL STUDIES OF PRACTICAL IMPORTANCE

A. OCCURRENCE OF AGGLUTININS AND MOUSE PROTECTIVE SUBSTANCES IN THE SERA OF ADULT SHEEP AND LAMBS

MATERIALS AND METHODS

Strain of Past. haemolytica. Bacterial suspensions for agglutination tests and for challenge purposes in mouse protection tests were prepared from a T strain, No. 7157H. Details of this strain are given in Appendix I.

Collection of Sera. Blood samples taken from apparently normal sheep and lambs of the Moredun Institute flock were allowed to clot for 3 hours. The sera were then separated, centrifuged and sterilised by filtration before storing at -30°C. None was inactivated by heat.

Agglutination Tests. These were performed according to the method described in Part 1. Doubling dilutions of serum ranging from 1/2 to 1/256 were used.

Passive Mouse Protection Tests. Mice were injected intraperitoneally with 0.5 ml. volumes of undiluted serum from the various animals under examination 5 to 6 hours before intraperitoneal challenge with organisms suspended in mucin. The techniques used were those already described in Part 1.

RESULTS

Previous observations had shown that all of 52 adult sheep of mixed ages possessed agglutinins to strain 7157H in their sera. The

titres rarely exceeded 1/32 and none was above 1/128. Of 19 three-month-old lambs tested 10 were completely negative.

A preliminary passive mouse protection test was performed to compare sera taken from (a) a 3-year-old sheep with an agglutinin titre of 1/32, and (b) a 3-month-old lamb possessing no agglutinins. Each serum was tested in 2 groups of 20 mice to allow for challenge with 2 dose levels. The results are recorded in Table 30 and show that the adult serum possessed marked protective properties in contrast to the lamb serum which failed to prevent very high mortality.

As a result of these findings an examination was made of sera taken from 20 adults whose ages ranged from 3½ to 6 years and 20 lambs between 4 and 5 months of age. Agglutination tests were carried out on all sera. The distribution of titres in lambs and adults is shown in Table 31. Fifty per cent of the lambs possessed no agglutinins. All adult sera were positive and their titres tended to be higher than those of positive lamb sera. Mouse protection tests were performed using sera from eleven of the lambs and ten of the adults. Three separate experiments were necessary and they were carried out within a period of 3 weeks. Two challenge levels were used and groups of mice consisted of either 6 or 8 animals. The results are given in Table 32 and it will be seen that the first and second experiments showed sera from 5 adults and 1 lamb to be markedly protective whereas sera from 5 lambs did not prevent high mortality in the mice. The results obtained in the third experiment were less clear-cut. Of 5 lambs and 5 adult sera only two appeared to be highly protective. These had both been collected from

TABLE 30

PASSIVE MOUSE PROTECTION TEST USING SERA FROM AN ADULT SHEEP AND A LAMB

Dose of viable organisms	Deaths produced by challenge in mice pretreated with normal adult or lamb serum	
	Adult serum	Lamb serum
79×10^6	$^2/_{20}$	$^{18}/_{20}$
39.5×10^6	$^0/_{20}$	$^{20}/_{20}$

Undiluted sera injected intraperitoneally in 0.5 ml. volumes.

Mice challenged intraperitoneally 5 to 6 hours later with strain 7157H in mucin.

TABLE 31

DISTRIBUTION OF AGGLUTININ TITRES TO STRAIN 7157H IN SERA OF 20 ADULT SHEEP AND 20 LAMBS

	Distribution of titres							
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Adult sera	0	0	1	2	5	6	4	2
Lamb sera	10	2	2	4	1	1	0	0

TABLE 32

PASSIVE MOUSE PROTECTION TESTS USING SERA FROM 10 ADULT SHEEP AND 11 LAMBS

		Deaths produced by challenge in mice pretreated with normal adult or lamb serum									
	Dose of viable organisms	Adult sera					Lamb sera				
		A1 (1/16)	A2 (1/64)	A3 (1/16)	.....	.....	L1 (0)	L2 (0)	L3 (0)	.....	.....
Exp. 1	54×10^6	$^1/_8$	$^0/_8$	$^0/_8$	.....	.....	$^8/_8$	$^0/_8$	$^7/_8$	.....	.....
	27×10^6	$^0/_8$	$^0/_8$	$^0/_8$	.....	.....	$^6/_8$	$^0/_8$	$^5/_8$	.....	.....
		A4 (1/16)	A5 (1/128)	.....	.....	.....	L4 (0)	L5 (1/16)	L6 (1/4)	.....	.....
Exp. 2	82×10^6	$^2/_8$	$^0/_8$	.....	.....	.....	$^8/_8$	$^8/_8$	$^8/_8$	.....	.....
	41×10^6	$^0/_8$	$^1/_8$	.....	.....	.....	$^3/_8$	$^8/_8$	$^7/_8$	.....	.....
		A6 (1/8)	A7 (1/64)	A8 (1/32)	A9 (1/32)	A10 (1/8)	L7 (0)	L8 (1/4)	L9 (1/2)	L10 (1/8)	L11 (0)
Exp. 3	64×10^6	$^5/_6$	$^0/_6$	$^1/_6$	$^5/_6$	$^3/_6$	$^3/_6$	$^5/_6$	$^6/_6$	$^5/_6$	$^3/_6$
	32×10^6	$^2/_6$	$^0/_6$	$^0/_6$	$^2/_6$	$^0/_6$	$^0/_6$	$^4/_6$	$^4/_6$	$^1/_6$	$^1/_6$

A = Adult serum.
L = Lamb serum.
Agglutinin titres given in brackets.
Undiluted sera injected intraperitoneally in 0.5 ml. volumes.
Mice challenged intraperitoneally 5 to 6 hours later with strain 7157H in mucin.

adults. It is clear from the results shown in the Table that the presence or absence of agglutinins did not correlate with the presence or absence of protective property.

B. INVESTIGATION OF CROSS-IMMUNOGENICITY BETWEEN STRAINS OF PAST. HAEMOLYTICA

Information on the technique of active mouse protection tests and on the classification of Past. haemolytica into A and T types has already been given. The present experiment consists of an investigation of the mouse-immunising ability of a number of A and T strains against challenge with a single T strain.

MATERIALS AND METHODS

Strains used for Vaccination. Groups of mice were vaccinated with the following A strains; Nos. 34, 70, 79, 97, 107, 109, 128 and 2054. Further groups were vaccinated with T strains Nos. 86, 105, 110, 123, 1118, 1190/1, 7157H and 2053. Details of these strains may be found in Appendix I.

Vaccination and Challenge Procedures. Intravenous vaccination was followed 9 days later by a further dose administered subcutaneously. Vaccinating doses consisted of 0.3 ml. volumes of 5 hour living broth cultures of the various strains. Such cultures of A and T strains were known to possess roughly comparable viable counts. A control group of mice received two similar dose volumes of sterile nutrient broth. Mice were challenged 12 days after having received the second dose of vaccine. Challenge was effected intraperitoneally using strain 7157H suspended in mucin. The technique of this procedure has already been described. Three dose levels were used and these were chosen on the basis of previous experience in order to give high mortality in control mice coupled with high protection in mice vaccinated with the challenge strain.

Mice. At the commencement of vaccination these were approximately 4 weeks old. Each group consisted of equal numbers of males and females.

RESULTS

Control mice were divided into 3 groups of 20 to allow for challenge with 3 dose levels. Mice vaccinated with the challenge strain also consisted of 3 groups of 20. Three groups of 10 mice were immunised with each of the other vaccine strains. All deaths occurred within 4 days of challenge. The results are shown in Table 33. The smallest challenge level produced 90% deaths in control mice while the 2 larger doses gave 100% mortality. Vaccination with the homologous strain provided very strong protection, much greater than that given by any of the heterologous strains. In fact the majority of heterologous strains gave no significant protection. A point of considerable interest emerges however if deaths produced by the two larger challenge doses are added. Accepting as the level of significance $p = 0.05$ six out of eight T strains appear to be immunogenically related whereas none of the A strains gave any protection against challenge with the T strain No. 7157H.

TABLE 33

INVESTIGATION OF CROSS-IMMUNOGENICITY BETWEEN STRAINS

		Deaths in controls	Deaths produced by challenge in mice vaccinated with various strains															
			T Strains								A Strains							
			7157H	86	105	110	123	1118	1190/1	2053	34	70	79	97	107	109	128	2054
Challenge doses of strain 7157H (viable organisms)	61×10^6	$^{20}/_{20}$	$^{12}/_{19}$	$^{9}/_{10}$	$^{6}/_{7}$	$^{8}/_{10}$	$^{8}/_{9}$	$^{10}/_{10}$	$^{10}/_{10}$	$^{8}/_{9}$	$^{10}/_{10}$	$^{10}/_{10}$	$^{9}/_{10}$	$^{9}/_{10}$	$^{10}/_{10}$	$^{10}/_{10}$	$^{10}/_{10}$	$^{10}/_{10}$
	30.5×10^6	$^{20}/_{20}$	$^{11}/_{20}$	$^{7}/_{10}$	$^{8}/_{10}$	$^{8}/_{10}$	$^{8}/_{10}$	$^{10}/_{10}$	$^{9}/_{10}$	$^{9}/_{10}$	$^{10}/_{10}$	$^{9}/_{10}$	$^{10}/_{10}$	$^{10}/_{10}$	$^{10}/_{10}$	$^{9}/_{10}$	$^{9}/_{10}$	$^{9}/_{10}$
	15.25×10^6	$^{18}/_{20}$	$^{3}/_{20}$	$^{5}/_{10}$	$^{9}/_{10}$	$^{9}/_{10}$	$^{8}/_{10}$	$^{6}/_{10}$	$^{8}/_{10}$	$^{9}/_{10}$	$^{10}/_{10}$	$^{8}/_{10}$	$^{9}/_{10}$	$^{9}/_{10}$	$^{10}/_{10}$	$^{10}/_{10}$	$^{10}/_{10}$	$^{9}/_{10}$

C. DEMONSTRATION OF MOUSE PROTECTIVE ANTIBODY IN SERA OF VACCINATED SHEEP

MATERIALS AND METHODS

Strain used for Vaccination and Challenge. The strain was No. 7157H details of which have already been given (see also Appendix I). A culture made from the heart blood of an experimentally infected mouse was freeze-dried and stored for use.

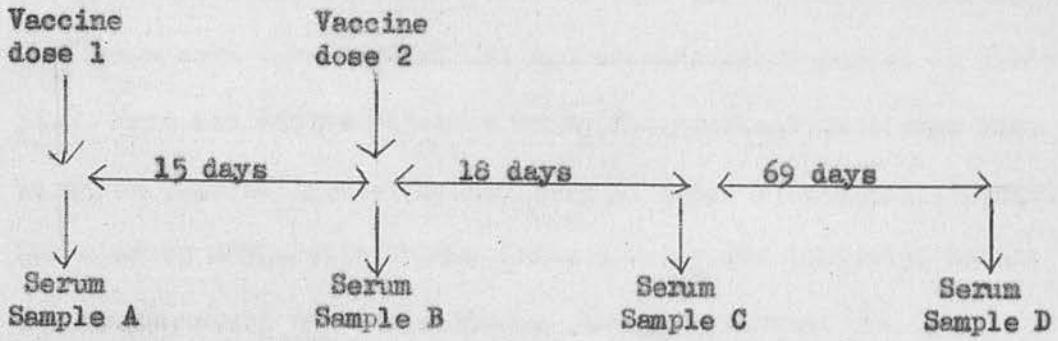
Preparation of Vaccine. 17 hour cultures on 5 per cent. sheep blood agar were suspended in normal saline and sterilised by washing twice and resuspending to the opacity of Brown's Tube 10 in 0.5 per cent formol-saline.

Sheep. These consisted of 15 two-year old breeding ewes.

Collection of Serum. On each occasion that serum was collected the method used was as follows. Volumes of approximately 50 ml. of blood were taken from each of the 15 sheep. These were allowed to clot for a few hours after which 15 ml. amounts of serum containing some red blood cells were removed from each and pooled. This pool of serum was centrifuged to remove red blood cells, sterilised by filtration and stored at -20°C until used. Shortly before use the serum was inactivated by heating for 30 minutes at 56°C.

Schedule for Vaccination and Serum Sampling. The time relationships

of these procedures are shown below.



Serum Samples A and B were withdrawn immediately before the injection of vaccine doses 1 and 2 respectively. The dose of vaccine per sheep was 8.0 ml. administered subcutaneously.

Agglutination Tests. These were performed by using 2-fold dilutions of serum and a formolised suspension of strain No. 7157H having the opacity of Brown's Tube 3.5. The tests were read after incubation in a water bath at 55°C for 2 hours.

Mouse Protection Test. This was carried out largely according to the method for performing passive intraperitoneal tests described earlier. Certain modifications were introduced however and an account of these is now given. Sheep serum was used undiluted in dose volumes of 0.5 ml. injected intraperitoneally. A 5 hour infusion broth culture supplied organisms for the purpose of challenge. The inocula consisted of mixtures of 1 part of bacterial suspension to 4 parts of 5 per cent mucin suspension inoculated intraperitoneally in dose volumes of 0.5 ml. 7 to 8 hours after the administration of serum. Three dose levels were used and these were shown by viable counts to contain 75, 37.5 and 18.7 million bacteria.

RESULTS

The abilities of serum pools A, B, C and D to protect mice from challenge were investigated and the results are recorded in Table 34. From the deaths obtained using the smallest challenge dose it is clear that the post-vaccinal Sera B, C and D were all protective for mice as compared with the Serum A which was collected before the commencement of vaccination. The odds against the least significant of these results having occurred by chance were approximately 80 to 1. The agglutinin titres of the serum pools A, B, C and D were 1/32, 1/512, 1/512 and 1/64 respectively.

TABLE 34

MICE CHALLENGED AFTER TREATMENT WITH SHEEP SERA COLLECTED BEFORE AND AFTER VACCINATION

Doses of viable organisms	Deaths in groups of mice pre-treated with sheep sera			
	Serum A	Serum B	Serum C	Serum D
75×10^6	$^{19}/_{19}$	$^{10}/_{10}$	$^{10}/_{10}$	$^{9}/_{10}$
37.5×10^6	$^{19}/_{19}$	$^{9}/_{10}$	$^{7}/_{10}$	$^{9}/_{10}$
18.7×10^6	$^{18}/_{18}$	$^{7}/_{10}$	$^{5}/_{9}$	$^{6}/_{10}$

DISCUSSION

PART 1

The lethal dose of living Past. haemolytica for mice by intravenous and intraperitoneal inoculation was shown to be a low multiple of the lethal dose of killed organisms. In view of this finding it seems unlikely that conventional methods of inoculation are capable of producing true infections in mice.

Mucin reduced by many millions the numbers of Past. haemolytica necessary to kill mice by intraperitoneal inoculation. This reduction represented a far greater number of bacteria than did the dose which, when combined with mucin, produced a high mortality. These considerations are of importance in establishing true infections with bacteria such as Past. haemolytica which are normally of low virulence for laboratory animals and whose lethal properties may well be due in part to the toxicity of the necessarily large inoculum. The dose of certain strains of Past. haemolytica required to produce a high death rate was still comparatively large even though mucin was used but the results recorded in Table 4 suggest that this did not apply equally to all strains. It was, however, well illustrated by No. 7157H, the strain which was used in all protection experiments. Nevertheless it is apparent that this method of challenge is likely to provide a satisfactory means of demonstrating and comparing protective potencies of various immune sera and of investigating cross antigenicity between strains in terms of immunising ability. A later experiment did in fact show that the passive intraperitoneal mouse protection test could be successfully used to demonstrate the production of immune antibody by vaccinated sheep.

Although the intracerebral method of challenge was capable of showing protection by hyperimmune rabbit serum, the demonstration of anything but gross differences in protective antibody content of sera by this means would not be possible without using large numbers of mice, and even this would probably be of no avail in attempted comparisons of sera of relatively low potency. Factors contributing to this state of affairs are the variation in susceptibility of mice to intracranial challenge, and the relative inefficiency of circulating rabbit antibody in combating cerebral infections with Past. haemolytica in mice. It must of course be borne in mind that rabbit antibody may be less efficient in this respect than that from other animal species.

Using intracerebral doses of organisms known from virulence titrations to be capable of producing high mortality, mice pre-treated with hyperimmune serum frequently showed increased survival times as compared with controls. This effect was seen especially over the first two or three days after challenge and was usually far more marked than actual protection from death. A number of factors may have been responsible for this, including the high initial concentration of circulating antibody and also the trauma produced by the inoculating needle which probably brought about some temporary increase in permeability of the blood-brain barrier. A further increase in this permeability probably occurred at a later stage from the infective process, but by this time the blood level of antibody was undoubtedly much reduced.

PART 2

Active immunity in mice was readily demonstrated by intraperitoneal challenge with organisms suspended in mucin and all of the vaccination procedures investigated were protective. No attempt was made to compare the immunising potencies of the various vaccines under test. Protection was assessed at certain times after vaccination without regard to measurement of the rise, fall and duration of immunity, or to the effect of dose of vaccine. In the case of comparative studies on routes of vaccination conclusions were drawn in strict relation to the particular experimental conditions used.

The active mouse protection test was designed to facilitate studies on immunity to Past. haemolytica, particularly on the immunogenic relationships between different strains and subsequent work demonstrated its success in this respect. The ease with which protective antibody could be demonstrated in the sera of vaccinated mice gave encouragement to a later investigation in which the mouse-protective abilities of pre-vaccinal and post-vaccinal sheep sera were compared.

While it is certain that humoral antibody was concerned in the immunity produced by vaccination, the possibility that non-specific protective mechanisms may also have played a part at the time of challenge was not eliminated. It is conceivable that substances giving rise to non-specific protection may be present in dead Past. haemolytica, and such a possibility cannot be adequately controlled in active protection tests. However, in view of the fairly long period between vaccination and challenge the effects of

such bacterial substances were probably of minor importance if indeed they existed at all. The same applies to any local peritoneal immunity which may have resulted initially from injecting the vaccine by the intraperitoneal route, and this is borne out by the fact that no significant difference could be observed between the efficiencies of intraperitoneal and intravenous vaccination against intraperitoneal challenge.

The degrees of protection afforded by intraperitoneal and subcutaneous vaccination differed strikingly, and this gives rise to speculation on the possible significance of route of vaccination against Past. haemolytica in the sheep.

Pfeiffer and Issaeff (1894) showed that certain substances such as broth and peptone solutions when injected intraperitoneally into guinea-pigs resulted in increased resistance to intraperitoneal challenge with cholera vibrios. Working with streptococcal infections in rabbits, Gay and Morrison (1923) found that the intrapleural injection of certain fluids, including broth, increased resistance to intrapleural challenge 24 hours later. This phenomenon was associated with an accumulation of macrophages in the pleural cavity. In the present study the protection against experimental Past. haemolytica infection produced by pretreatment of mice with fluids, including normal saline and dilutions of normal mouse serum, was considerable. This protection was easily obliterated by increasing the challenge doses however and these increased challenge doses were known from previous work to have been readily capable of demonstrating true passive antibody immunity. These observations have considerable practical importance in studying

immunity against Past. haemolytica infections in mice. In passive protection tests in which serum dilutions are injected into the peritoneal cavity a few hours before intraperitoneal challenge one must use challenge doses large enough to overcome the non-specific resistance produced in control mice. In active immunity experiments smaller challenge doses are satisfactory.

PART 3

The first of a series of pathogenicity experiments in young adult sheep confirmed the conclusions of most earlier workers as to the high resistance of these animals to large doses of Past. haemolytica administered intratracheally. Primary cultures and diseased lung suspensions were used as inocula to ensure that organisms had not lost virulence and to cover the possibility of multiple aetiology involving inapparent agents such as viruses and pleuropneumonia-like organisms. The results were completely negative.

Intravenous inoculations of sheep were carried out using a strain derived from a case of lamb septicaemia. Doses of hundreds of millions of bacteria were necessary to ensure death. Detailed observations on three sheep showed that the disease was characterised by a diphasic temperature reaction. At the first peak symptoms of illness were slight or absent but with the second temperature rise they appeared and increased in severity until the time of death. There is little doubt that the first temperature rise was due to the presence of pyrogen in the inocula since it could be reproduced as a monophasic reaction by inoculating broth culture filtrate or 10 ml. of killed broth culture and also by inoculating living culture into

a sheep protected by large doses of streptomycin. The subject of bacterial pyrogens has been reviewed by Bennett and Beeson (1950). These substances are commonly produced by Gram-negative bacteria and are not affected by the methods of sterilisation in general use.

The disease resulting from intravenous inoculation was of short duration with symptoms of respiratory distress and diarrhoea and a post-mortem picture characterised by congestion, oedema and haemorrhage. This is the type of disease which, according to Topley and Wilson (1955), might be expected to result from the presence of toxic concentrations of a Gram-negative organism. Death with somewhat similar symptoms and lesions was in fact produced by the intravenous injection of heat-killed broth culture in a dose which, in terms of bacterial generations, was not far removed from the large dose of living organisms necessary to ensure fatal infection.

Examination of three sheep inoculated intravenously showed that the numbers of organisms in the blood stream tended to be low and at times blood cultures were negative. At about the time of death however a definite sharp increase in numbers was observed which suggested a sudden release of bacteria from some heavily loaded site. This increase may have been due to contraction of the spleen. The spleen contained massive numbers of bacteria at the time of death whereas other sites examined showed comparatively few. In each case the number of organisms in the spleen exceeded that which had been inoculated by many thousands of millions, but in view of the large infecting dose this cannot be taken as proof of a high degree of multiplication.

It is interesting that the distribution of Past. haemolytica

in the organs of a 7-month-old lamb which died in the field from septicaemia was different from that observed in sheep which died from experimental infections. In the natural case the lungs and liver contained the largest numbers of viable organisms. This difference may have resulted from variations in susceptibility related to age. Certainly Past. haemolytica septicaemia is rarely encountered in animals more than a year old.

Tweed and Edington (1930) produced a rapidly fatal haemorrhagic disease in calves by intravenous inoculation, and the septicaemic disease of lambs was reproduced by Stamp et al. (1955) and Biberstein and Kennedy (1959). A feature common to all these experiments was the use of infecting doses which were large, so that probably after only a few bacterial generations toxic levels of organisms were reached. Nevertheless in lambs the pathological picture seen in field cases of septicaemia could be almost exactly reproduced by intravenous inoculation of such doses. It is highly unlikely that animals are exposed to these large numbers of organisms under field conditions and lambs which succumb to the natural disease are probably those whose resistance is lower than average due either to stress or to some intrinsic factor or both.

PART 4

Montgomerie et al. (1938) described two serological types of Past. haemolytica but their clear-cut results could not be confirmed by Bosworth and Lovell (1944). Florent and Godbille (1950) also differentiated two types by serological methods. The work of Carter (1956a) on the other hand suggested that a large number of strains,

including two from lamb septicaemia in Scotland, were serologically homogeneous. Biberstein et al. (1958) reported two types of Past. haemolytica, one of which was thought to be a variant of the other. The two types differed colonially and serologically but were biochemically indistinguishable.

The occurrence of two types of Past. haemolytica as recorded in this thesis appears to be a different phenomenon from that reported by Biberstein et al. Numerous differential criteria were established in vitro and on examination of a considerable number of diseased ovine carcases it was found that all cases of septicaemia in lambs aged 6 to 12 months were associated with Type T infections. All cases of enzootic pneumonia and also the great majority of lambs which died from septicaemia within the first few weeks of life yielded Type A strains. A small number of enzootic pneumonia cases were encountered in which mixtures of A and T organisms occurred.

It seems unlikely that one type arises as a spontaneous variant of the other since the differences between them are probably too numerous to be genetically linked and in addition both A and T strains appear to be perfectly stable in the laboratory. In spite of the numerous differences between the two types it should be pointed out that superficially they bear a very close resemblance to each other and this fact probably explains why they have not been recognised in the past.

In a limited number of trials in which the pathogenicity of A and T strains was tested by intravenous inoculation in sheep and lambs no differences in behaviour between the two types could be detected. Very large doses of bacteria sometimes produced death but the numbers

of organisms present in various sites after death did not indicate extensive multiplication.

It should be mentioned that a later experiment designed to investigate cross-immunogenicity between a number of Past. haemolytica strains in mice suggested that the majority of T strains tested shared a common antigen which produced a low grade immunity. None of the A strains tested appeared to possess this antigen.

It is now clear that the organisms investigated by Stamp et al. in their work on lamb septicaemia were of the T variety. Several of their strains, Nos. 1087/3, 1118, 1127 and 1190/1 have in fact been identified as such in the present studies.

PART 5

Stamp et al., while considering that they had established the causal relationship of Past. haemolytica to the septicaemic disease, drew attention to the widespread doubt which exists as to the pathogenic significance of the organism in enzootic pneumonia. The possible aetiological involvement of a filterable virus has been suggested by a number of workers including Montgomerie et al. (1938), Salisbury (1957) and Downey (1957). This suggestion has arisen chiefly as a result of the high resistance of sheep and laboratory animals to experimental Past. haemolytica infections, particularly those of the respiratory tract. The view that a virus may be involved is not supported however by the almost universal failure to reproduce enzootic pneumonia by inoculating diseased tissue suspensions from field cases.

The present experiments on pathogenicity in young lambs have

conclusively shown that under certain circumstances Past. haemolytica can be of very high virulence for the ovine species. A freshly isolated Type A strain derived from a 3-week-old lamb which died of a generalised infection was capable of producing death in young lambs when inoculated in small doses. Marked bacterial multiplication occurred in vivo. Intraperitoneal inoculation of extremely small or larger dose levels produced rapidly fatal peritonitis and intravenous inoculation of larger doses gave similar results. This type of disease occurs in the field. Small intravenous doses resulted in prolonged infections which were frequently fatal and various lesions were produced, all of which are at times encountered in natural cases. These lesions sometimes included pneumonia and it is interesting to recall that Stamp et al. in dealing with the septicaemic disease expressed the view that in some cases where pneumonic lesions were present they appeared to be secondary to bacterial thrombi in the pulmonary vessels. Intratracheal inoculations of larger doses also produced pneumonia. Adult sheep were included in the experiments involving intraperitoneal and intravenous inoculation and these animals completely resisted doses which proved fatal to lambs. Since some of the sheep used were only twelve months old it appears that this high degree of immunity develops within the first year of life. The much lower virulence for lambs of the T strain, 7157H, by intraperitoneal inoculation may well have resulted from repeated subculture in the laboratory.

PART 6

Agglutinins against the T strain, No. 7157H, were found in the

sera of all adult sheep tested, usually in low titres, but were frequently absent in the sera of lambs. The work of Bosworth and Lovell (1944) demonstrated that Past. haemolytica could be isolated from the upper respiratory tracts of a proportion of apparently healthy sheep but it is not known at present whether this applies to both types A and T. It is nevertheless tempting to suggest that the widespread occurrence of serum agglutinins in adults arises as a result of contact with the organism. Any assumptions on this point would however be unwarranted in view of the work of Gibson (1930), Lovell (1932) and Lovell (1934) which showed that the sera of a number of animal species, including the sheep, contained agglutinins to a wide range of bacteria many of which were not known to be associated with these species.

It was also found in a limited number of experiments that sera taken from apparently normal lambs and sheep varied in their abilities to protect mice from challenge with the T strain, No. 7157H. The protective power of sera did not correlate with the occurrence of agglutinins. In general adult sera were considerably more protective than lamb sera but exceptions and intermediate results occurred. It is of interest to refer once more to the experiment discussed earlier in which adults were completely resistant to doses of an A strain which proved fatal to a high proportion of very young lambs.

Carter (1956a) using haemagglutination and agglutination tests found that strains of Past. haemolytica were serologically homogeneous and pointed out the importance of such information for the proper selection of cultures in vaccine production. In the present investigations

an active mouse protection test was carried out in order to study cross-antigenicity between a number of A and T strains in terms of immunising ability. All mice were challenged with a single T strain and a vaccine prepared from the homologous organism gave a high degree of immunity. The majority of T strains appeared to share an immunising antigen of minor importance. It produced only a low grade immunity despite the administration of two doses of vaccine. Vaccines composed of A strains gave no demonstrable immunity against the challenge organism. It is apparent that Past. haemolytica is not an immunogenically homogeneous species.

The passive mouse protection test was used successfully to demonstrate the increased protective potency of sheep sera following vaccination. The need for some such indirect immunological test results from the high cost of the sheep as an experimental animal and also from the difficulties associated with producing experimental infections in sheep. The existence of correlation between the mouse protective and sheep protective properties of antibodies produced in sheep by vaccination remains to be proved. The high susceptibility of very young lambs to experimental infection with Past. haemolytica gives hope that a direct method of assessing immunity may be possible. As always, however, the final test of any immunising agent must be its successful use under field conditions.

SUMMARY

Immunological work has hitherto been severely limited by the high resistance of laboratory animals and sheep to reasonably small doses of Past. haemolytica. This has also led to speculation as to the pathogenic significance of the organism in sheep disease.

The present studies showed that true infections could be established in mice by two methods, namely (a) intracerebral inoculation, and (b) intraperitoneal inoculation of organisms suspended in mucin. Either method when used as a means of challenge was capable of demonstrating passive immunity. For this purpose however the second procedure was the more successful and it was also very satisfactory for demonstrating active immunity produced in mice by different vaccination procedures. Subcutaneous vaccination was less effective than immunisation by the intraperitoneal or intravenous routes. Immunity could be passively transferred by injecting serum from vaccinated mice into non-vaccinated mice. Procedures leading to non-specific changes in mouse-resistance were investigated.

Very large doses failed to produce disease in sheep by intratracheal inoculation but were sometimes fatal when injected intravenously. The implications of using such doses are discussed in the light of experiments on toxicity of the organism. Invasiveness of Past. haemolytica in experimentally and naturally infected sheep was investigated.

Each strain of the organism examined was classified as one of two Types, designated A and T. Although closely similar these two Types were distinguishable by certain in vitro characteristics.

Cases of enzootic pneumonia in sheep and of septicaemia in very young lambs were associated with Type A infections while septicaemias of older lambs yielded Type T organisms.

Three-week-old lambs were highly susceptible to extremely small doses of an A strain which were completely resisted by young adult sheep. Intraperitoneal inoculation resulted in rapid death from peritonitis. Intravenous inoculation frequently produced prolonged infections with many of the lesions seen in field cases, including pneumonia. Pneumonia was also produced by intratracheal inoculation of larger doses.

Low titre agglutinins to a T strain were found in the sera of all adult sheep tested but were frequently absent in lambs. By means of the passive mouse test it was found that normal adult sera tended to be more protective than normal lamb sera against challenge with a T strain.

Using the active mouse protection test it was shown that the species Past. haemolytica is not immunogenically homogeneous. It also appeared that the majority of T strains examined shared an immunising antigen of minor importance. This antigen was not possessed by A strains.

The use in sheep of a formol-saline vaccine prepared from a T strain stimulated the production of antibody which was capable of protecting mice from challenge with the homologous organism.

REFERENCES

Bennett, I. L., and Beeson, P. B. (1950). Medicine, Baltimore, 29, 365.

Beveridge, W. I. B. (1937). Aust. vet. J., 13, 155.

Biberstein, E. L., Meyer, Margaret E., and Kennedy, P. C. (1958). J. Bact., 76, 445.

Biberstein, E. L., and Kennedy, P. C. (1959). Amer. J. vet. Res., 20, 94.

Bosworth, T. J., and Lovell, R. (1944). J. comp. Path., 54, 168.

Campbell, D., Campbell, R. S. F., and Dromey, L. (1949). Vet. Rec., 61, 64.

Carter, G. R. (1954). Can. J. comp. Med. vet. Sc., 18, 359.

Carter, G. R., and McSherry, B. J. (1955). Ibid., 19, 177.

Carter, G. R. (1956a). Can. J. Microbiol., 2, 483.

Carter, G. R. (1956b). Can. J. comp. Med. vet. Sc., 20, 374.

Downey, N. E. (1957). N. Z. vet. J., 5, 128.

Dungal, N. (1931). J. comp. Path., 44, 126.

Evans, D. G., and Perkins, F. T. (1954). J. Path. Bact., 68, 251.

Florent, A., and Godbille, M. (1950). Ann. Med. vet., 94, 337.

Gale, C., and Smith, H. R. (1958). Amer. J. vet. Res., 19, 815.

Gay, F. P., and Morrison, L. F. (1923). J. infect. Dis., 33, 338.

Gibson, H. G. (1930). J. Hyg., 30, 337.

Hamdy, A. H., and Pounden, W. D. (1959). Amer. J. vet. Res., 20, 78.

Hamdy, A. H., Pounden, W. D., and Ferguson, L. C. (1959). Ibid., 20, 87.

Hartley, W. J., and Boyes, Betty W. (1955). Proc. N. Z. Soc. anim. Prod., 15, 120.

Jones, F. S. (1921). J. exp. Med., 34, 561.

Leyshon, W. J. (1932). Vet. J., 88, 256.

Live, I. (1949). Amer. J. vet. Res., 10, 347.

Lovell, R. (1932). J. comp. Path., 45, 27.

Lovell, R. (1934). Ibid., 47, 107.

Lovell, R., and Hughes, D. L. (1935). Ibid., 48, 267.

McFarland, J. (1907). J. Amer. med. Ass., 49, 1176.

Miles, A. A., and Misra, S. S. (1938). J. Hyg., Camb., 38, 732.

Montgomerie, R. F., Bosworth, T. J., and Glover, R. E. (1938). J. comp. Path., 51, 87.

Morley, D. C. (1945). J. Path. Bact., 57, 379.

Newsom, I. E., and Cross, F. (1932). J. Amer. vet. med. Ass., 80, 711.

Olitzki, L. (1948). Bact. Rev., 12, 149.

Pfeiffer, R., and Issaeff, - (1894). Z. Hyg. InfectKr., 17, 355.

Pittman, Margaret (1931). J. exp. Med., 53, 471.

Rosenbusch, C. T., and Merchant, I. A. (1939). J. Bact., 37, 69.

Salisbury, R. M. (1957). N. Z. vet. J., 5, 124.

Sawyer, W. A., and Lloyd, W. (1931). J. exp. Med., 54, 533.

Spray, R. S. (1923). J. infect. Dis., 33, 97.

Stamp, J. T., Watt, J. A. A., and Thomlinson, J. R. (1955). J. comp. Path., 65, 183.

Stevens, A. J. (1957). Vet. Rec., 69, 1249.

Topley, W. W. C., and Wilson, G. S. (1955). Principles of Bacteriology and Immunity, 4th Ed., 2, 1151.

Tweed, W., and Edington, J. W. (1930). J. comp. Path., 43, 234.

Woxholtt, C., Naerland, G., and Hoff, H. (1952). Nord. VetMed., 4, 433.

The following publications record certain aspects of work described in the Thesis.

Smith, G. R. (1958). Experimental Infections of *Pasteurella haemolytica* in Mice and their use in demonstrating Passive Immunity. J. comp. Path., 68, 455.

Smith, G. R. (1959). The Production in Mice of Active Immunity against *Pasteurella haemolytica*. J. comp. Path., 69, 116.

Smith, G. R. (1959). Isolation of Two Types of *Pasteurella haemolytica* from Sheep. Nature, 183, 1132.

Smith, G. R. (1959). The Pathogenicity of *Pasteurella haemolytica* for Young Lambs. J. comp. Path. (in press).

APPENDIX I

Strains of Past. haemolytica used in Experiments

The strains described below were isolated from sheep and lambs in Scotland unless stated otherwise. The cases of enzootic pneumonia resembled those described by Montgomerie et al. (1938) and the septicaemias were similar to those recorded by Stamp et al. (1955). All strains produced narrow zones of haemolysis on sheep blood agar and showed the morphology of Past. haemolytica on microscopic examination. They were indol negative, nitrate positive and gave slight growth when incubated on McConkey medium. Fermentation reactions were carried out in the medium recommended by Bosworth and Lovell (1944). Positive reactions were given in xylose, glucose, fructose, mannose, sucrose, maltose, dextrin, mannitol and sorbitol. Gas was not produced. Inulin, erythritol, adonitol and dulcitol were not fermented. Mice resisted doses of 0.1 ml. of 6 hour broth cultures injected intraperitoneally. Strains were classified as types A or T by methods described in the thesis.

No. 8 Isolated Oct. 1955 from nasal swab of apparently healthy sheep. Type A.

No. 10 Isolated Oct. 1955 from nasal swab of apparently healthy sheep. Type A.

No. 32 Isolated Dec. 1955 from enzootic pneumonia in S. Wales. Type A.

No. 34 Isolated Jan. 1956 from enzootic pneumonia. Type A.

No. 36 Isolated March 1956 from nasal swab of apparently healthy sheep. This was the predominating organism. Type A.

No. 70 Isolated May 1956 from enzootic pneumonia. Type A.

No. 79 Isolated June 1956 from enzootic pneumonia. Type A.

No. 82B Isolated Aug. 1956 from enzootic pneumonia. Type A.

No. 86 Isolated Sept. 1956 from lamb septicaemia. Type T.

No. 89B Isolated Oct. 1956 from pneumonia in which C. pyogenes was the predominating organism. Type T.

No. 91 Isolated Oct 1956 from enzootic pneumonia. Type A.

No. 92 Isolated Oct. 1956 from enzootic pneumonia. Type A.

No. 94 Isolated Dec. 1956 from lamb septicaemia. Type T.

No. 95 Isolated Dec. 1956 from enzootic pneumonia. Type A.

No. 96 Isolated Dec. 1956 from enzootic pneumonia. Type A.

No. 97 Isolated Dec. 1956 from enzootic pneumonia. Type A.

No. 99 Isolated Jan. 1957 from enzootic pneumonia. Type A.

No. 100C Isolated Jan. 1957 from enzootic pneumonia. Type T. (A Type A organism was also isolated from the same lung).

No. 103 Isolated Jan. 1957 from enzootic pneumonia. Type A.

No. 105 Isolated Sept. 1957 from lamb septicaemia. Type T.

No. 107 Isolated Feb. 1958 from enzootic pneumonia. Type A.

No. 108 Isolated April 1958 from enzootic pneumonia. Type A.

No. 109 Isolated May 1958 from enzootic pneumonia. Type A.

No. 110 Isolated Nov. 1958 from lamb septicaemia on Bass Rock. Type T.

No. 112 Isolated Nov. 1958 from enzootic pneumonia. Type A.

No. 114a Isolated Nov. 1958 from enzootic pneumonia. Type A.

Nos. 121, 123, 124, 125. Isolated Nov. 1958 from different outbreaks of lamb septicaemia in Kent. Type T.

No. 128 Isolated Dec. 1958 from enzootic pneumonia. Type A.

No. 140 Isolated April 1959 from enzootic pneumonia. Type A.

No. 158 Isolated May 1959 from septicaemia in a 3-week-old lamb. Type A.

Nos. 1087/3, 1118, 1127, 1190/1. Strains isolated and used by Stamp et al. (1955) in investigation of lamb septicaemia. Type T.

No. 2050 Isolated Oct. 1958 from enzootic pneumonia. Type A. (No. 2051, Type T, was also isolated from the same lung).

No. 2051 See above.

No. 2053 Isolated Sept. 1958 from enzootic pneumonia. Type A. (No. 2054, Type T, was also isolated from the same lung).

No. 2054 See above.

No. 2056 Isolated Sept. 1958 from enzootic pneumonia. Type T. (A Type A organism was also isolated from the same lung).

No. 2058 Isolated Sept. 1958 from enzootic pneumonia. Type T. (A Type A organism was also isolated from the same lung).

No. 7157H Isolated Sept. 1954 from lamb septicaemia. Type T.

APPENDIX II

Method of Performing Viable Counts

Ten-fold dilutions of bacterial suspensions were made up in 1% casein hydrolysate solution by transferring 0.1 ml. of suspension into 0.9 ml. of diluent, using a separate 1 ml. pipette for each dilution. A dropping pipette calibrated to deliver 0.02 ml. per drop (Miles and Misra, 1938) was used to sample each dilution, the drops being placed on the surfaces of sectored blood agar plates. Colonies were counted after 18 hours' incubation. A number of replicate counts was usually performed and the average taken as the true value.